Proceedings
of the
Society
for
Experimental Biology and Medicine

VOL. 88 MARCH, 1955 No. 3

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Xanthurenic Acid* Excretion Studies in Diabetics With and Without Retinopathy.† (21576)

DAVID A. ROSEN, GERTRUDE D. MAENGWYN-DAVIES, BERNARD BECKER, HOWARD H. STONE, AND JONAS S. FRIEDENWALD. (Introduced by Joseph L. Lilienthal, Jr.)

From the Wilmer Ophthalmological Institute, Johns Hopkins Hospital and University.

In studies(1) carried out on diabetic patients with and without retinopathy, it has been observed that 40% of diabetics without retinopathy fail to respond by a significant eosinophil drop to an intramuscular dose of 25 mg of corticotropin. Diabetics with retinopathy respond uniformly to this dose, as do healthy non-diabetic subjects. It has been noted repeatedly that non-reactive diabetics can be made to show an eosinophil drop to this dose of corticotropin by prior intramuscular administration of 100-300 mg of pyridoxine hydrochloride. We therefore considered it desirable to investigate whether diabetics with and without retinopathy differ in their nutritional status with reference to this vitamin. Certain other vitamins are also capable of restoring the eosinopenic response of the diabetic to a normal level and it is not implied that the defect in eosinopenic response is specifically a symptom of pyridoxine deficiency. The present investigation, however, concerns tests for the presence or absence of pyridoxine deficiency in diabetics and controls.

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* The authors acknowledge the technical assistance of Mary Steigner and Heidi Duda and are indebted to Dr. Jessie W. Huff of Sharp and Dohme Co. for the xanthurenic acid.

† This work was supported by a grant from the National Institutes of Health, Division of Neurological Diseases and Blindness.

Materials and methods. Diabetic and non-diabetic subjects were derived from the clinic and ward services of the Wilmer Institute of the Johns Hopkins Hospital. The diagnosis of diabetic retinopathy was established after dilatation of the pupils with 10% neosynephrine. The retinopathy varied in severity from the presence of occasional capillary aneurysms to retinitis proliferans. Patients were given an oral test dose of 10 g dl-tryptophane dispersed in fruit juice with the aid of a Waring blender, following which a complete 24 hour urine specimen was collected under toluene as a preservative. Xanthurenic acid output was determined according to the method described by Wachstein and Gudaitis(2). Since this method was found to be most sensitive in the range of 150 to 300 γ of xanthurenic acid, we added 200 γ of xanthurenic acid dissolved in ethanol to each 1 to 4 ml aliquot of the 24 hr urine. A Coleman Junior Spectrophotometer at a wave length of 610 millimicra was used for the color determinations. In order to obtain comparable blanks, an equal volume of ethanol not containing xanthurenic acid was added to each blank. All determinations were run in duplicate.

Results: (Table I).

TABLE I. Urinary Excretion of Xanthurenic Acid (mg in 24 Hr) after Oral Test Load of 10 g dl-tryptophane.

Group:	1 Non-diabetics	2 Diabetics without retinopathy	3 Diabetics with retinopathy
	12.02	26.86	32.02
	0.00	54.23	47.52
	3.83	12.70	19.12
	10.78	41.20	41.63
	13.25	27.95	54.02
	20.35	17.58	84.26
	4.36	13.59	24.60
	13.48	6.06	15.36
			29.70
			41.20
			45.90
			23.35
Mean	$\bar{X}_1$ 9.76	$\bar{X}_2$ 25.02	$\bar{X}_3$ 38.22
	$S\bar{X}_1$ 5.76	$\bar{X}_{2,3}$ 32.94	
		$S\bar{X}_{2,3}$ 3.74	

$\bar{X}$ = mean; $S\bar{X}$ = stand. error of mean.

Comparing the diabetics with the normal controls the difference is found to be significant on the Student t-Test ($P < .01$). Comparing diabetics with and without retinopathy the difference is found not to be significant ($P > .05$).

To test whether the xanthurenic acid excretion was influenced by pyridoxine, 6 cases were chosen whose excretion of xanthurenic acid after a test dose of tryptophane was 35 mg or greater. These patients were then given pyridoxine and after this retested. The results are shown in Table II. It is evident that the xanthurenic acid excretion of these patients is markedly reduced following the administration of pyridoxine.

TABLE II. Reversal by Pyridoxine of Excess Urinary Excretion of Xanthurenic Acid (mg/24 Hr) after Oral Test Load of 10 g dl-tryptophane.

		Xanthurenic acid	
Patient	Diabetic retinopathy	Before pyridoxine	After pyridoxine
MFW	+	35.00	24.58
CW	—	47.60	15.04
AA	+	36.36	27.20
GH	—	43.00	20.00
ED*	—	37.80	18.20
ML†	—	213.20	5.70

All these patients were given an intramuscular injection of 300 mg of pyridoxine before being retested.

* ED when retested after a single intramuscular dose of 300 mg pyridoxine excreted 39.3 mg xanthurenic acid. He was then given 500 mg of pyridoxine orally every day for one wk and one further intramuscular dose of 300 mg before being retested. The figure shown in Table is the excretion of this patient after this prolonged treatment.

† Patient ML was given 5 injections of 300 mg pyridoxine intramuscularly before being retested.

Discussion. The data summarized in Tables I and II indicate that many diabetics with and without retinopathy are at least marginally deficient in pyridoxine. Though the diabetics without retinopathy excreted on the average more xanthurenic acid than did those with retinopathy, the difference was not statistically significant. Kotake and Tani(3) reported finding xanthurenic and 3-hydroxykynurenic acids in the urine of diabetic subjects whose only source of tryptophane was in their regular diet. Urines from normal subjects did not show these materials on paper chromatograms. They did not divide their patients on the basis of retinopathy status.

Becker(4) and Friedenwald(5) have pointed to the possibility that diabetics who develop retinopathy may have a higher level of adrenal cortical activity than those who do not, and that this adrenal overaction may be operative in the pathogenesis of the retinal lesions. Stebbins(6) and Dean and Shaw (7) reported that vit. B_6 deficiency in rats is associated with histological adrenal cortical atrophy, and evidence of underactivity of this gland. We consequently investigated the possibility of inducing pyridoxine deficiency in diabetics with retinopathy, hoping thereby to lower the activity level of their adrenal cortex. We employed the metabolic antagonist desoxypyridoxine by subcutaneous injection in doses of 100 mg every other day, and used enough of this material (2 to 8 doses) to produce increased xanthurenic acid output. In no instance were we able to detect any influence on adrenal cortical function as manifested by 11-oxysteroid excretion, or eosinophil response to ACTH. Furthermore, in no instance was the retinopathy influenced. These results are in accord with those of Vilter and co-workers(8) who found no alteration in adrenal cortical function in subjects made clinically deficient in vitamin B_6 by use of desoxypyridoxine.

Conclusions and summary. 1. Diabetic patients with and without retinopathy excrete on the average significantly greater quantities of xanthurenic acid after an oral test load of 10 g dl-tryptophane than do non-diabetic controls. 2. There is no significant difference in the xanthurenic acid output of diabetic patients who have, and those who have not developed retinopathy.

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Received June 11, 1954. P.S.E.B.M., 1955, v88.

Organ Plasma Volume of Normal Unanesthetized Mice.* (21577)

J. J. FRIEDMAN. (Introduced by H. S. Mayerson.)

From the Biophysics Laboratory and Department of Physiology, School of Medicine, Tulane University, New Orleans, La.

In order to analyze translocations of plasma between tissues of the mouse during the development of experimental traumatic shock, it is necessary to know the normal plasma volume of the tissues. In other studies on organ plasma volume of the mouse(1,2), the use of anesthesia, the method of killing the animals, the dissection technic and the method of sampling blood were such that significant variations in the tissue plasma volume measurements were unavoidable. This report represents an attempt to obviate these limitations in carrying out measurements of organ plasma volume in the mouse.

Method. The plasma volumes of various tissues in the mouse were determined by injecting the mice with radioiodinated albumin and assaying the tissue samples for radioactivity. The mice used were male albinos of the CF-1 strain, weighing 19-23 g. Human serum albumin[†] labelled with radioactive iodine (I-131)[‡] was prepared according to the method of Wasserman and Mayerson(3) and was injected intravenously in volumes of 0.1 ml.

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* Material taken from a dissertation submitted by Julius Jay Friedman to the Graduate School of Tulane University in partial fulfillment of requirements for degree of Doctor of Philosophy.

The animals were sacrificed by rapid immersion in liquid nitrogen, with a temperature of approximately –195°C. According to Stone (4) a mouse is frozen solid in about 2 seconds following immersion in liquid nitrogen, thus making it possible to obtain tissue samples from the frozen animal instantaneously and simultaneously without the use of anesthesia. Samples of liver, kidney, spleen, and lung were easily obtained free of tissue debris; the intestines sometimes contained undigested material; the muscle often included excess fatty tissue; and the skin always included subcutaneous tissue. Central blood samples were obtained by permitting the heart to thaw and then discarding all solid debris, leaving whole blood behind. After sampling, the tissues were weighed and digested in alkali according to the method of Fine and Seligman(5) preparatory to assaying for radioactivity. The plasma volume of the tissues was determined as the ratio of radioactivity per unit weight of tissue to the radioactivity per unit weight of plasma. The radioactivity per unit weight of plasma was calculated from the hematocrit and the radioactivity per whole blood sample. The hematocrit of the blood sample was determined by means of a gradient density column(6). The gradient column was established by partially mixing proportions of Xylene and Carbon Tetrachloride necessary to provide a continuous density range from 1.040 to 1.080. The density of a drop of hemolyzed blood was read against Potassium Sulfate density standards.

Results. Following intravenous administration, radioactive iodinated albumin leaves the circulation in a uniform exponential manner for 1-1½ hours. The disappearance constant (K) expressed as the fractional disappearance of albumin from the plasma per minute is –0.00636 for this phase. In order for circulatory mixing to be complete an interval of 10 minutes was permitted to elapse following injection before sampling. Longer post-injection periods were avoided since approximately 25% of the iodinated albumin is removed from circulation within the first hour.

TABLE I. Wet Weight of Mouse Organs.

	Wt in g Mean ± S.E.	Range
Whole animal	21.000	
Liver	1.675 ± .056	1.510-1.960
Kidney	.390 ± .008	.371- .416
Spleen	.106 ± .007	.084- .135
Lung	.148 ± .001	.121- .164
Intestine	1.541 ± .061	1.411-1.648
Skin	2.700 ± .067	2.449-2.915
Muscle*	8.380 ± .243	7.592-9.219

* Difference in carcass wt before and after alkali digestion.

Table I lists the mean unit weights of the various organs, the range of values, and the standard error. The averages are of 10 mice and compare favorably with those reported previously(1).

Table II lists the mean plasma volumes of the various organs, expressed as ml per gram of tissue along with the range of values, the standard error and the per cent of total plasma volume. Included for comparison are the results previously reported by Kaliss and Pressman(1) in which mice were sacrificed with chloroform. Many of the discrepancies in the data reported by Kaliss and Pressman (1) and those presented in this paper may be attributed to the use of anesthesia(7).

Discussion. Variations in the estimation of organ blood volumes are not unusual in view of sensitivity of the peripheral vascular elements to nervous and humoral influences which are operating continuously to maintain a homeostatic internal environment. The handling of the animal, the use of drugs, hemorrhage, and manipulation of tissue all result in a disturbance of the normal physiologic activity of the animal by producing significant translocations of blood between the tissues via vasoconstriction and/or vasodilatation. Since most of these undesirable influences are obviated by freezing animals in li-

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[†] We are grateful to the American Red Cross for supplying the albumin used.

[‡] Radioactive iodine was obtained from Isotopes Division of the Oak Ridge National Laboratory, Oak Ridge, Tenn.

TABLE II. Plasma Volume of Mouse Organs.

Tissue*	Plasma vol (ml/g) Mean ± S.E.	Plasma vol (ml/g) Range	% total plasma vol per organ	Kaliss & Pressman(1) ml plasma/g	Kaliss & Pressman(1) % total plasma vol
Whole animal	.051 ± .002	.045-.066	100.0	.067	100.0
Liver	.134 ± .003	.098-.188	21.8	.202	23.0
Kidney	.085 ± .002	.065-.116	3.2	.191	4.8
Spleen	.077 ± .003	.042-.128	.8	.092	.5
Lung	.302 ± .007	.247-.384	4.4	.274	2.6
Intestine†	.034 ± .001	.029-.044	5.1	.050	
Skin	.020 ± .001	.010-.032	5.3		
Muscle	.024 ± .001	.008-.037	19.6	.016	

* Avg of 30 mice. † 15 mice.

quid nitrogen, we prefer to regard the data reported here as being more representative of normal organ plasma volume than data which has been reported in the literature.

Human serum albumin was used as the tracer vehicle because of its availability. The mouse does not appear to exhibit any species specificity to serum albumin, for both rabbit and bovine albumin have been used without any reaction or difference in tissue blood volume measurements(1). In this investigation, the mice, observed for as long as 15 hours post injection of human serum albumin, displayed no ill effects.

Summary. The plasma volume of various mouse organs was determined without the use of anesthesia by injecting mice with radioiodoalbumin, freezing them in liquid nitrogen and assaying tissue samples for radioactivity. Values are presented for plasma volume, expressed as ml per gram of tissue, of the liver, kidney, spleen, lung, intestine, skin, and muscle.

The helpful criticism of Dr. H. S. Mayerson and Dr. J. K. Hampton, Jr. is gratefully acknowledged.

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Received June 23, 1954. P.S.E.B.M., 1955, v88.

Serum Iron Levels in Rhesus Monkeys with Chronic Vitamin C Deficiency.* (21578)

LOUIS D. GREENBERG AND JAMES F. RINEHART.
From Department of Pathology, University of California School of Medicine, San Francisco.

During the course of some earlier studies on the serum iron levels of rhesus monkeys on control, and on diets deficient in various vitamins, hypoferremia was observed in 2 monkeys in a state of chronic ascorbic acid deficiency. A search of the literature revealed that Braganca *et al.*(1) had observed that a marked decrease in plasma iron accompanied scorbutic anemia in the guinea pig. The present paper represents an investigation of the alterations in serum iron and the concomitant changes in the blood picture in 2 additional monkeys with chronic ascorbic acid deficiency and also includes observations on the effect of the administration of iron alone and in combination with ascorbic acid on the hypoferremia and upon the anemia. After this work had been completed, a paper by Proehl and May

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* This investigation was aided by a grant from the National Vitamin Foundation. We are grateful to Lederle and Co., Pearl River, N. Y. for supplies of folic acid.

(2) came to our attention which reported decreased serum iron levels in monkeys subjected to milk diets deficient in ascorbic acid and low in folic acid.

Methods. Six rhesus monkeys whose initial weights were 2.3-3.0 kilos were used in the present studies. They received the same basal diet, supplements, care, and handling described in an earlier publication(3) with the exception that the customary daily 25 mg allotment of ascorbic acid (AA) was omitted from the vitamin tablet of 4 animals subjected to the deficient regimen. In order to maintain the monkeys alive and in a state of chronic deficiency after they reached the acute stage, each received 1 mg dose of AA by mouth either daily or thrice weekly as necessary. Periodically, blood specimens were drawn by venipuncture for hematological examination and for the estimation of serum iron. In order to avoid inconsistencies due to diurnal variations in serum iron, blood was taken in the morning at approximately the same hour each time. Hemoglobin was determined by the method of Sahli and serum iron was measured by a modification of the method of Burch *et al.*(4). It differed from the original procedure merely in the use of 20-fold larger volumes of serum and reagents and in the application of the Coleman Model 6 spectrophotometer instead of the Beckman DU. Some supplementary tests were carried out on plasma AA with the micro-method of Butler and coworkers(5) and on serum alkaline phosphatase with the procedure of Armstrong and King as modified by King(6). Two animals were maintained on the control diet with adequate AA throughout the experiment. In addition, each experimental animal served as his own control with respect to the effect of AA deficiency on the serum iron and on the blood picture and with respect to the alterations induced by the administration of iron and AA. Only 2 of the chronically deficient macaques survived for the entire course of the experiment. For this reason the data presented are primarily limited to the 2 survivors.

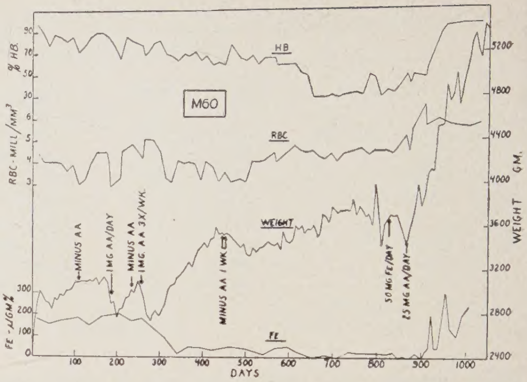

FIG. 1. Monkey No. 60. Alterations in HB, RBC, WT and serum FE.

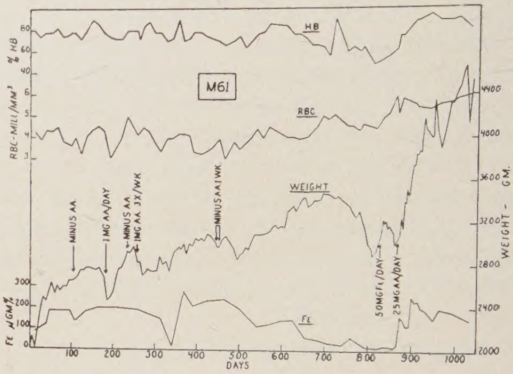

FIG. 2. Monkey No. 61. Alterations in HB, RBC, WT and serum FE.

Results. Fig. 1 and 2 show the effects of withdrawal of AA on the serum iron (FE), weight (WT), red blood cells (RBC) and the hemoglobin (HB) of 2 monkeys. It will be observed that approximately 65-80 days after withdrawal of the vitamin the 2 animals began to lose weight rapidly and developed all the manifestations of acute scurvy, notably hemorrhages, painful joints, extreme weakness, etc.; however, the FE values remained essentially unchanged except for some minor fluctuations. Following administration of small amounts of AA, monkey 60 showed a much more rapid decline in FE than did monkey 61, but both animals finally attained levels generally below about 60 γ %. The HB and RBC declined also, but not until the monkeys had been on the low AA intake for a considerable time. After the FE levels had remained low for a number of weeks, 50 mg of iron as ferrous sulfate were administered *per os* daily for an extended period. Even after 41 days of this massive iron therapy there was no increase in the FE, although a slight to moderate increase in HB and RBC occurred. Finally,

TABLE I. Serum Alkaline Phosphatase.

Period	King-Armstrong units Avg	Range
Control		
#60	60.6 (4)*	52.4-68.5
61	59.7 (4)	53.2-67.6
Chronic AA deficiency (450-800th day)		
#60	30.4 (22)	21.1-42.0
61	39.5 (23)	22.6-72.0

* No. of determinations.

with the iron intake remaining at 50 mg per day, the AA supplement was increased to 25 mg daily. In a matter of weeks the 2 monkeys showed a marked increase in FE, HB, and WT. The response in the RBC was somewhat variable; monkey 60 exhibited a substantial augmentation while monkey 61 showed only a slight increase.

The average and mean deviation of 90 estimations of the FE of the control animals and for 8 determinations on monkeys 60 and 61 made prior to withdrawal of AA are, respectively: 185 ± 50 and 160 ± 29 γ %. The average value for the control monkeys is in excellent agreement with that reported by Proehl and May(2) who employed an entirely different analytical procedure. Additional data were obtained on a third chronically deficient monkey who exhibited a marked hypoferremia and anemia between the 365th and the 600th day of the experiment. The average and mean deviation of 11 measurements of the FE for this period were computed to be 37 ± 20 γ %.

The marked increase in weight which occurred in monkeys 60 and 61 (Fig. 1 and 2) following the introduction of an adequate intake (25 mg) of AA should provide ample evidence that these monkeys were suffering from a deficiency of this vitamin, since it is the only known factor required by the monkey (vit. B_{12} was not provided but it has not been proven indispensable) which was omitted or fed in inadequate amounts. This is substantiated by our observations on serum alkaline phosphatase (Table I). Comparison of the average serum alkaline phosphatase values for the control period and for the period of chronic deficiency extending from the 450th to 800th day of the experiment shows that the average values during the latter period were decreased from the corresponding control values by 50% in #60 and by 34% in #61. Although a reduction in serum alkaline phosphatase is not specific for scurvy, nevertheless it is a valid supplementary criterion for ascorbic deficiency when employed in conjunction with other criteria. Several investigators(7-9) have demonstrated a reduction in the serum alkaline phosphatase of scorbutic guinea pigs. Plasma AA was also followed during the early part of the experiment and a definite decrease in the concentration could be demonstrated during the first 2 months of deficiency, but in view of the fact that the monkeys exhibited such low levels on an adequate intake with a decrease to near 0 values during the first 2 months of the experiment, further estimation of the plasma AA was considered unwarranted. The average of 16 determinations during the control period was 0.27 mg % (0.15-0.40) and for 8 determinations during the second month of deficiency 0.06 mg % (0.04-0.12).

Discussion. It is amazing that despite the daily administration of 50 mg of extradietary iron *per os* to each of the 2 chronically deficient monkeys for a period of 41 days (a total of over 2 g of iron to each), the low FE levels persisted without any alteration until AA was provided in adequate amounts. It is not known whether the defect in iron metabolism in scurvy is related to impaired absorption from the gastrointestinal tract, defective utilization or to loss of iron from the body. Our experiment throws no light on the mechanism of this defect. Evidence exists in the literature in support of the idea that AA increases iron absorption(10-13). Gorten and Bradley(14) have postulated that the vitamin has an effect upon utilization of iron.

Proehl and associates(2) have observed low FE levels in monkeys subjected to a diet deficient in AA and low in folic acid. Although their observations were limited to a single determination approximately one week after therapy, these authors found the FE levels of 3 hypoferremic monkeys to be unaffected by a single intravenous administration of 30-50 mg of iron. From analyses of FE, erythrocyte protoporphyrin and the urobilinogen excretion, they favored the concept that the anemia and hypoferremia of scurvy result from blood loss via hemorrhage, although they did not exclude impairment of absorption or utilization of iron from playing a role. The experiments of Proehl and May do not represent simple, uncomplicated AA deficiency, since their milk diets were inadequate in folic acid as well as AA and as a consequence some of their control monkeys developed manifestations of folic acid deficiency(15).

Bronte-Stewart(16) observed low FE, low total iron-binding capacity and anemia in a number of cases of adult human scurvy. Complete hematological response occurred in these subjects following the administration of AA alone, and intravenous iron alone failed to influence the bone marrow or the blood picture. Bronte-Stewart attributed the low FE to the presence of one or more intramuscular hematomata in his patients. Infections and sterile abscesses(17-19) are known to produce hypoferremia. In the case of our monkeys, no obvious infections or hemorrhages were present although minor hemorrhages into the joints cannot be ruled out.

It is rather unlikely that the hypoferremia observed is the result of chronic infection, since Tötterman(20) has shown that the anemia of infection is quite refractory to therapy either with AA or iron alone or in combination. In view of the marked HB increase observed in the 2 scorbutic monkeys following therapy with iron and AA concomitantly, their low FE levels do not appear to be related to the anemia of infection.

Summary. The serum iron levels and blood picture were followed in 2 rhesus monkeys with chronic vitamin C deficiency. Hypoferremia and anemia were observed in both monkeys. The oral administration of iron alone had no influence on the hypoferremia and produced only a slight to moderate increase in the RBC and HB. Combined therapy with both iron and ascorbic acid brought about marked improvement in the hypoferremia and anemia.

We wish to express our appreciation to Miss Julie van Snellenberg and Mrs. Margot Smyth for their technical assistance.

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Received November 22, 1954. P.S.E.B.M., 1955, v88.

Threshold Studies on Production of Experimental Epilepsy with Alumina Cream.* (21579)

William H. Faeth, A. Earl Walker, Alberto D. Kaplan, and Wilbert A. Warner.

From Division of Neurological Surgery, Johns Hopkins University School of Medicine, Baltimore, Md.

The unpredictable production of experimental epilepsy in the monkey using various alumina cream compounds prompted an investigation to find a preparation giving consistent and reproducible results. From available evidence it seemed that there should be a quantitative relationship between the amount of alumina and the production of seizures.

Method—materials. Preparation of alumina cream. The alumina(3) was prepared in the following manner: To 1000 cc of a 1% solution of ammonium alum ($NH_4Al(SO_4)_2 \cdot 12H_2O$) was added, with constant stirring, 800 cc of a 1% solution of ammonium hydroxide (NH_4OH), at room temperature; thus forming a white precipitate of aluminum hydroxide ($Al(OH)_3$) which rapidly settled out of solution. Additional ammonium hydroxide (the total never exceeding 1000 cc) was added until precipitate was no longer formed. The entire colloidal suspension was repeatedly washed with distilled water until sulfate ions could no longer be detected in the supernatant liquid by testing with barium acetate. The suspension was then centrifuged at 3000 r.p.m. for 2 hours and the supernatant fluid decanted off yielding a thick cream having a total volume of 50 cc. Analysis of this preparation revealed: a) *pH* of 8.0-8.1 (determined by glass electrode) b) *Aluminum*—18-22 mg/cc (determined by precipitating the aluminum as the hydrated oxide by means of ammonia solution in the presence of ammonium chloride, the gelatinous precipitate thus formed being washed, converted into the oxide by ignition, and weighed as Al_2O_3) c) *Sulfate ion*—less that 9.6 mg/cc (determined by qualitative precipitation with lead) d) *Ammonia*—none detectable by microKjeldahl technic. Two cubic centimeter aliquots of the preparation were then sealed in glass ampules. Even dispersion of the cream was insured by vigorous shaking of the ampules prior to use. *Injection.* Twenty-four monkeys (*Macaca mulatta*) averaging 3-4 kg were prepared, under intravenous pentobarbital anesthesia, with bilateral trephinations over the primary motor arm area (area 4) leaving the dura intact. Various quantities of the standard alumina preparation were then injected through the dura mater with a 20 gauge hypodermic needle and tuberculin syringe, angled in such a fashion as to insure the deposition of the alumina in cortical grey matter. The quantities utilized and the number of monkeys receiving each quantity are represented graphically in Fig. 1. *Electroencephalography.* Serial electroencephalographic tracings with and with Metrazol (pentamethylentetrazol) activation were made at regular intervals throughout the observation period. Frontal, central, temporal and occipital leads were placed bilaterally and linked for recording by bipolar technic with an eight-channel

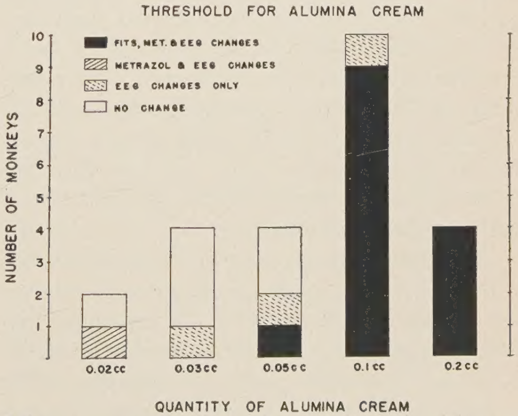

FIG. 1. Graph illustrates relationship between quantity of alumina cream injected and incidence of production of seizures.

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* This work was aided by a grant from the National Institute of Neurological Diseases and Blindness, National Institutes of Health, U. S. Public Health Service.

Model III Grass electroencephalograph. *Metrazol activation* with known sub-convulsive doses (0.2-0.3 cc of 10% solution) was routinely employed(4) during recording but only those animals in which actual clinical seizures were produced have been listed as demonstrating increased sensitivity to the drug.

Results. As indicated in Fig. 1, it became apparent that a definite quantitative threshold existed for the production of seizures from the motor cortex of monkeys with a standard preparation of alumina cream. Ninety percent of those animals receiving injections of 0.1 cc of the preparation developed spontaneous fits; while only 10% of those receiving lesser amounts of the same material had attacks. Those which were injected with larger amounts invariably developed seizures. Electroencephalographic abnormalities were found in all animals in which 0.1 cc or more was utilized, whereas less than half of those injected with smaller quantities showed similar changes. Increased sensitivity to Metrazol activated seizures was demonstrated in only 2 of 10 animals with less than 0.1 cc of alumina cream, while 13 out of 14 who had received 0.1 cc or more showed activation of the electroencephalogram and developed convulsive phenomena with the drug.

The spontaneous seizures which developed consisted, in the main, of focal tonic and clonic fits, usually beginning in the upper limb contralateral to the side of the injection, and frequently showed a typical Jacksonian march. Less commonly the attacks were unilateral or generalized at the onset, and usually were of 15-19 seconds duration. Two members of the series developed uncontrollable attacks and died in status epilepticus. In addition to spontaneous fits, most of the animals could be provoked into seizure by vigorous physical stimulation.

The electroencephalographic abnormalities consisted chiefly of focal abnormalities—isolated spikes, bursts or runs of continuous discharges—arising from the motor cortex at the site of injection. Less frequently, delta range activity and occasionally merely sharp waves were observed. The presence of a low voltage fast "mirror" focus on the contralateral motor cortex was a common finding particularly in those animals observed for a long period of time.

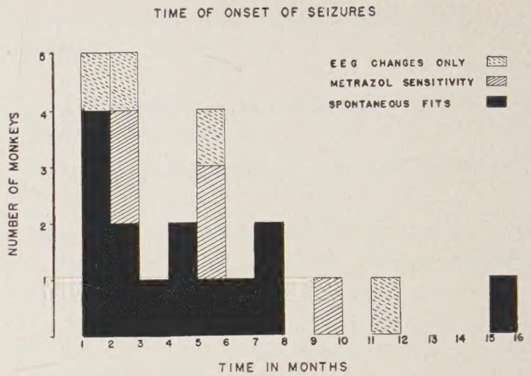

FIG. 2. Graph illustrates time of onset of seizures following injection with alumina cream. Note initial latent period which averaged 6 wk.

The development of seizures usually followed a delay of at least 6 to 7 weeks; the length of this delay bearing no relationship to the quantity of alumina injected. Fits and/or EEG abnormalities were seen to occur as early as two months and as late as 16 months after injection, but in most instances developed within 6 months, as illustrated in Fig. 2. The total period of observation varied from 4 months to 2 years. Those animals which failed to develop seizures were observed for an average period of one year following injections.

Discussion. The value of alumina cream in the production of experimental epilepsy is once again confirmed. The technic of intracortical injection employed here differs from the method of disk application utilized by Kopeloff *et al.*(6), Pope(7), Cure(1), Walker (9) and their co-workers. Since it is felt that the direct contact between the epileptogenic agent and the neurons is responsible for the production of convulsive manifestations, the intracortical injection method was felt to be superior for the purpose of this study. The injection technic has been previously shown to be effective in the hands of such investigators as Sloan(8), Kennard(5) and Gastaut(2), even when applied to structures at subcortical levels. It remained only to be shown that a quantitative relationship existed between the amount of alumina cream employed and the actual production of seizures.

Conclusions. 1. There appears to be a quantitative threshold for production of chronic experimental epilepsy in the monkey with intracortical injection of a standardized preparation of alumina cream. 2. The level of this threshold for the motor cortex of the *Macaca mulatta* is 0.1 cc of alumina cream, containing 18-22 mg of aluminum/cc.

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Received December 14, 1954. P.S.E.B.M., 1955, v88.

Inactivation of MEF1 Poliomyelitis Virus by Ionizing Radiation. (21580)

ERNEST C. POLLARD AND LISBETH M. KRAFT. (Introduced by David Weinman.)

From Biophysics Division, Sloane Physics Laboratory and Department of Microbiology, Yale University.

Inactivation of viruses by ionizing radiation has been much studied(1). Use of heavily ionizing radiation such as alpha particles and deuterons enables a measurement of effective cross section, which, as determined by observing the slope of the straight line obtained by plotting the logarithm of the survival ratio against the dose, has been found in many cases to be correlated either with the full size of the virus, or with that of some specially sensitive internal unit.

This paper is concerned with the loss of infectivity under deuteron and alpha particle bombardment. While this work was in progress a report of alpha particle bombardment of the Lansing strain of poliomyelitis virus was presented by Bonet-Maury and Latarjet(2).

Materials and methods. The MEF1 baby mouse adapted strain of Type 2 poliomyelitis virus was used throughout. For desiccation, cover slips (½ in. diam. circles) were fixed with silicone grease into the 10 depressions of a circular brass plate and sterilized by dry heat. Five hundredths ml of a 20% baby mouse brain suspension containing virus in 15% Bacto-peptone(3) was spread evenly over the surface of each cover slip. This was rapidly frozen at –70°C on a level surface. The plate holding the cover slips was designed to permit irradiation of dry virus under vacuum in a laboratory not ordinarily concerned with pathogenic material. The depressions containing the cover slips were connected by indents to holes that lead to the outside and that were filled with absorbent cotton which would permit evacuation of the system without any possible contamination coming through to the outside. This first plate was then covered by 2 more which kept in position a thin aluminum foil of thickness equivalent to 1 cm of air in such a way that only the foil stood between the specimen and the irradiating beam. This meant that at no time would the virus be exposed in the physics laboratory and yet bombardment by fast particles would be possible. After bombardment, the virus-containing cover slips receiving the same dose of deuterons or alpha particles as well as those serving as controls (no radiation) were placed in tubes to which enough distilled water was added to make a 10^{-1} concentration of brain. To insure best possible contact between the water and the

TABLE I. Results of Bombarding MEF1 Poliomyelitis Virus with Deuterons.

$D \times 10^{11}$	$2.3 \log \frac{n}{n_o}$	$S\ (\times 10^{-12}\ cm^2)$
1.13	1.84	16.3
1.17	0	0
1.17	1.15	9.8
1.31	3.0	22.8
1.7	0	0
2.05	1.38	6.9
2.13	.23	1.0
2.26	.92	4.1
2.5	1.61	6.4
2.72	1.85	6.8
2.76	1.84	6.7
3.25	.69	2.1
3.75	1.60	4.3
4.4	1.38	3.1
4.75	2.06	4.4
4.75	3.0	6.3
5.5	3.0	5.4
6.6	2.75	4.2
11.3	4.6	4.1

Avg $S = 6.0 \times 10^{-12}\ cm^2$
$\sigma = \pm 5.3 \times 10^{-12}\ cm^2$

dry material, cover slips were fragmented with the end of a pipette which remained in the tube during the reconstitution period of 30-60 min. at 4°C. Thus the particles of dry material that gained access to the pipette during maceration of the cover slips were equally reconstituted. Beginning with the 10^{-1} dilution, sufficient serial ten-fold dilutions were made in physiological saline to reach a titration endpoint, and 0.02 ml of each dilution was inoculated intraspinally into 6-8 adult Swiss mice. Animals were observed for 10 days for paralysis and death. Titers of samples were calculated according to the method of Reed and Muench(4).

Results. The cross sections resulting from several bombardments are presented in Tables I and II. Table I corresponds to bombardment by deuterons and represents the sum total of 5 runs, while Table II lists the results of 4 runs using alpha particles. The inactivation appears to follow the semi-logarithmic relation, $\ln\left(\frac{n}{n_o}\right) = -SD$, as predicted for any statistical inactivation by randomly distributed particles(1,3), where $\frac{n}{n_o}$ is the survival ratio and D is the number of particles per cm^2. The values of the cross section, S, scatter somewhat as indicated by the rather large standard deviation, but there seems to be no reason to disbelieve the general logarithmic trend.

The figure for the cross section, S, of the infective virus obtained for the case of deuterons is $6.0 \pm 5.3 \times 10^{-12}\ cm^2$. In the case of alpha particles the figure obtained is $7.1 \pm 3.5 \times 10^{-12}\ cm^2$. If the cross section obtained for alpha particle bombardment is taken to represent the projected area of a sphere of radius r, the radius derived is 15.7 ± 4.8 mμ and the diameter is 31.4 ± 9.6 mμ. This fits quite well with the recently reported electron micrograph figures for poliomyelitis virus (27-30 mμ(5) and 28 mμ(6)). The fact that although deuterons ionize about one-fourth as densely as alpha particles and yet produce an inactivation approximately as great, argues that the sensitive volume is moderately homogeneous. The fact that the cross sections derived from both types of radiation are large indicates that the infective virus is very sensitive to these densely ionizing radiations. The sensitivity is such that the whole virus, or very nearly the whole virus, appears to take part in infectivity and that the sensitive unit is at least relatively thick.

Summary. Baby mouse adapted MEF1 poliomyelitis virus has been bombarded by deuterons and alpha particles. The inacti-

TABLE II. Results of Bombarding MEF1 Poliomyelitis Virus with Alpha Particles.

$D \times 10^{11}$	$2.3 \log \frac{n}{n_o}$	$S\ (\times 10^{-12}\ cm^2)$	$r\ (\times 10^{-12}\ cm)$
1.24	.69	5.6	1.29
1.43	.92	6.4	1.43
2.13	2.76	12.9	2.03
2.7	2.52	9.4	1.73
2.96	.69	2.32	2.72
3.56	4.8	13.3	2.06
4.1	2.06	5.1	1.27
4.3	1.84	4.3	1.17
4.95	5.5	11.1	1.69
5.46	2.52	4.7	1.21
6.4	5.3	8.3	1.62
6.5	1.16	2.5	.89
7.1	6.45	9.1	1.71
7.2	2.99	4.2	1.15

Avg $S = 7.1 \times 10^{-12}\ cm^2$
$\sigma = \pm 3.5 \times 10^{-12}\ cm^2$
Avg r = 15.7 mμ
$\sigma = \pm 4.8$ mμ

vation of infectivity corresponds to a cross section of $7.1 \pm 3.5 \times 10^{-12}$ cm^2 for alpha particles and $6.0 \pm 5.3 \times 10^{-12}$ cm^2 for deuterons. The radius of an assumed spherical sensitive volume so deduced is 15.7 ± 4.8 mμ.

We express our thanks to Mr. Dwight Wilson for assistance in running the cyclotron, Mr. S. Person for the design and aid in construction of the bombardment arrangements, and Miss Nancy Smale for technical help.

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Received January 13, 1955. P.S.E.B.M., 1955, v88.

Effect of Ethylenediamine Tetracetic Acid, Magnesium and Calcium on Succinate and Keto Acid Metabolism.* (21581)

DAVID B. TYLER.

From Department of Pharmacology, School of Medicine, University of Puerto Rico, San Juan.

Ethylenediamine tetracetic acid (EDTA) protects some enzyme systems against inactivation that occurs during the preparation or incubation of the tissues(1-7). Its action in some cases is indirect due to its ability to bind and remove inhibiting metal ions(1,4); in others, there is evidence for a direct effect independent of its chelating ability(2,3,5). The reports of the effect of EDTA on succinate oxidation are conflicting. Under some conditions, it activates(4,5); under others, it appears to inhibit(1) the succinoxidase system. This paper is concerned primarily with the mechanism of action of EDTA on succinate oxidation and to compare the effects of this agent (with and without added metal ions) with those produced by DPN and ATP. Since oxalacetate (OAA), formed during succinate oxidation, has a profound inhibitory action on the succinoxidase system(8,9), particular attention is given to the effect of the experimental conditions on the production and utilization of this keto acid.

Methods. Isotonic NaCl, containing an excess of cytochrome c to which various additions were made, was used as a medium. Phosphate, Tris- (hydroxymethyl) aminomethane, or glycylglycine were used as buffers

TABLE I. Effect of EDTA and DPN on Succinate Oxidation (Oxygen Uptake) and Keto Acid Accumulation (Figures in Parentheses). Warburg experiments. Reaction volume 2 ml. All flasks contained in final concentrations: Phosphate (.01 M), succinate (.02 M), cytochrome c (5×10^{-5} M). Other additions as indicated. 0.5 ml of 5% whole homogenate (0.4-0.9 mg N) added to all vessels. Keto acids expressed as micromols OAA/mg N. O_2 consumption corrected for endogenous respiration and expressed as μl/mg N/hr. Temp. 38°C; gas phase, air; duration, 60 min. See text for levels of differences between groups.

	No Mg^{++}			Added Mg^{++} (.003 M)		
	Cont.	EDTA (.001 M)	DPN (.001 M)	Cont.	EDTA (.001 M)	DPN (.001 M)
Heart	220 (.28)	110 (.79)	110 (.65)	250 (.24)	240 (.29)	150 (.87)
Kidney	160 (.13)	90 (.68)	100 (.49)	170 (.12)	170 (.08)	130 (.42)
Liver	150 (.17)	70 (.53)	140 (.17)	160 (.13)	120 (.21)	140 (.66)
Brain	110 (.13)	60 (.22)	80 (.22)	130 (.15)	140 (.14)	90 (.23)

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* This work was supported by grants from Public Health Service, National Institute of Neurological Diseases and Blindness, and from Life Insurance Medical Research Fund. Preliminary report presented at 1954 Meetings of Am. Physiol. Soc. at Madison.

(pH 7.4). Five per cent whole tissue homogenates or mitochondrial suspensions were prepared in the cold as previously described (10). The usual Warburg procedures were employed for determining the rate of succinate oxidation(10), all O_2 data being corrected for endogenous respiration. Generally, the substrates were tipped in from the side arm to the enzyme and the other components of the medium after 4 minutes for temperature equilibration and initial readings were taken 1 minute later; in some experiments the substrates were added after the enzyme was incubated for various intervals of time. Here readings were started 1 minute after such additions. In other experiments the enzyme was added directly to the medium containing all the substrates. Where respiration was not measured, a Dubnoff shaking incubator was used, the ice-cold enzyme being added to the complete medium and immediately incubated. Any deviation from these procedures are described below. Keto acid accumulation was measured(11) by determining the total amount present after one hour of succinate oxidation. This data is given as micromoles of OAA.† Utilization of OAA was calculated (11) from the amount that disappeared in 15 or 20 minutes after the enzyme was added to a medium containing a measured quantity of this keto acid. (Under these experimental conditions, those tissues with the highest rate did not deplete the added keto acid by more than 60-70%.) Zero time determinations were made in both types of studies. The reaction was stopped by adding an equal volume of ice cold 10% trichloracetic acid to the contents of the vessel. Other pertinent details of the experimental procedures are given in the figures and tables. Where the results of a typical experiment are given it is taken from a large series of similar runs. The level of significance of the differences reported is given below.

TABLE II. Effect of Ca^{++}, Mg^{++} and Mn^{++} on Keto Acid Accumulation and OAA Utilization by Kidney Homogenates. Data for keto acid accumulation averages from 15 experiments; for OAA utilization (conc. start = .0015 M), from 18 experiments with phosphate buffer and 15 with Tris buffer. In 6 experiments the same tissue preparation was used for comparison of phosphate and Tris buffers under conditions represented in lines 1, 3, 5 and 6 and columns e to j. Concentrations as given in Table I.

		Keto acid accumulation (μM/mg N/hr) phosphate buffer				OAA utilization (μM/mg N/hr) phosphate buffer				OAA utilization (μM/mg N/hr) Tris buffer	
Line No.	Additions (final conc.) M	Cont. (a)	EDTA (b) (.001 M)	DPN (c) (.001 M)	ATP (d) (.002 M)	Cont. (e)	DPN (f) (.001 M)	ATP (g) (.002 M)	EDTA (h) (.001 M)	Cont. (i)	EDTA (j) (.001 M)
1	None	.14	.94	.89	.46	2.61	3.19	3.79	3.11	3.41	6.63
2	Ca^{++} .0003	—	—	—	—	1.10*	—	—	4.10†	1.90*	7.02
3	" .0006	.10	.29*	.56*	.19*	1.25*	1.90*	2.61†	3.79	1.97*	7.23†
4	" .003	—	—	—	—	1.67†	—	—	1.06*	1.51*	1.90*
5	" .03	—	—	—	—	2.88	2.28†	2.88	2.24	1.56*	1.78*
6	Mg^{++} .003	.11	.11*	1.24*	.76*	6.83*	5.97*	7.73*	6.07*	7.20*	7.46†
7	Mn^{++} "	.05†	.08*	.49*	.14*	7.65*	9.15*	9.10*	—	—	—
8	Ca^{++} & .0003 Mg^{++} .0015	.09	.06*	.60*	.19*	2.81	5.00*	6.15*	—	—	—
9	Ca^{++} & .0003 Mg^{++} .006	.06†	—	.52*	—	3.72†	7.43*	—	—	—	—

* Differences between experimental groups and controls in each vertical column significant at 1% level. † Significant at 5% level.

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† OAA was a neutralized solution made immediately before use from a supply prepared from sodium ethyl oxalacetate. DPN "9OL", ATP (sodium salt), Tris, and cytochrome c were obtained from Sigma Chemical Co.; EDTA ("Sequestrene" obtained from Alrose Chemical Co.) was a 3X recrystallized disodium salt used in a neutral solution.

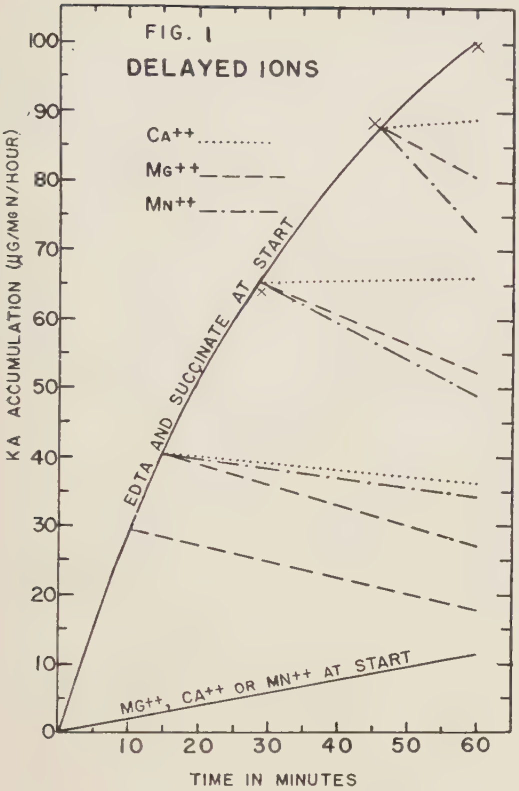

FIG. 1. Averages from 22 experiments for EDTA and succinate: 9 for Mg^{++}; 9 for Ca^{++}; and 4 for Mn^{++}. When a pair of vessels containing metal ions in side arms were tipped, another control pair containing trichloracetic acid in side arms were tipped and immediately removed from bath. The first pair continued to incubate until time indicated. Main compartments contained kidney homogenate, phosphate buffer, succinate, cytochrome c and EDTA as given in Table I. Metal ions (final concentration): Mg^{++} (.003 M), Ca^{++} (.0015 M), Mn^{++} (.003 M).

Results. A. *Keto acid accumulation during succinate oxidation.* In the absence of added metal ions, the addition of EDTA (final concentration = .001 M) increases markedly the amount of keto acids found at the end of 60 minutes of succinate oxidation (Table I, figures in parenthesis). The percentage increase is much greater in kidney, liver and heart than in brain (400, 200, 180 and 70%, respectively). All these differences are significant at the 1% level. At the same time EDTA also inhibits by about 50% succinate oxidation (differences significant at 1% level). The addition of Mg^{++}, Ca^{++} or Mn^{++} completely prevents the stimulating effect of EDTA on keto acid accumulation (see also Table II) and simultaneously abolishes its inhibitory action on succinate oxidation. Note that Ca^{++} and Mg^{++}, when added together, do not antagonize one another on this action of EDTA (Table II, column b, line 8). The results of experiments where the ions are added after various intervals to an incubating reaction media containing EDTA, succinate and the enzyme indicate that the accumulation of keto acids ceases immediately on such additions, then, depending upon the metal ion added, the concentration may remain stationary for the rest of the incubation period, as occurs with Ca^{++}, or may fall off as is found with Mg^{++} or Mn^{++} (Fig. 1).

Preincubating the enzyme (aging) for one hour before adding the EDTA and succinate also abolishes the stimulating effect of EDTA on keto acid accumulation (Fig. 2, inserted figures in shaded columns), indicating a requirement for this action of EDTA of a substance easily inactivated or depleted by aging the enzyme. DPN is regarded as the labile substance required by malic dehydrogenase for OAA production(12). Therefore, it is surprising that brain, a tissue rich in DPNase (13,14), appears the most resistant to aging. Also as a result of aging the enzyme, EDTA is no longer very effective in inhibiting succinate oxidation (Fig. 2), indicating that the inhibitory action of EDTA on the succinoxidase system is associated with its ability to increase OAA formation.

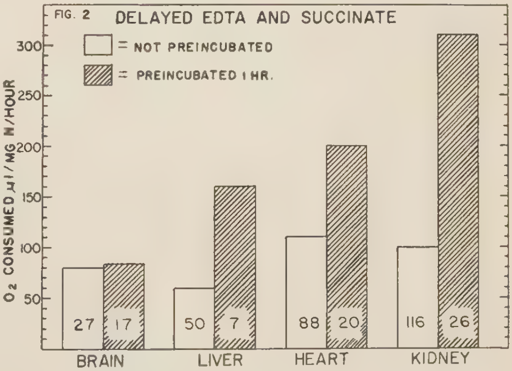

FIG. 2. Typical experiment on tissues from same animal. EDTA and succinate added to "not preincubated" after 4 min. for equilibration; to "preincubated" after 1 hr. Other conditions as in Table I.

TABLE III. μM Keto Acid (as OAA) Found by Fresh and Preincubated Tissue Preparations 1 Hr after Addition of Succinate. These results on preparations of pooled kidneys of 2 rats are typical of 4 others. Mitochondria separated from 30 ml of homogenate used in "A" and resuspended in 15 ml saline. Fresh whole homogenates added to flasks within 5 min. after death; fresh mitochondria within 60 min. Preincubated preparation shaken at 38°C in buffered saline before succinate and other additions were made except line 11 where EDTA was present. Dubnoff shaker; concentrations as in Table I.

Line #		A Whole homogenates	B Mitochondria
1	Fresh + succinate	.11	.07
2	*Idem* + ATP	.32	.11
3	" + EDTA	.69	.17
4	" + DPN	1.0	.44
5	" + " + EDTA	1.5	.57
6	Preincubated 1 hr, then succinate added	.05	.05
7	*Idem* + ATP	.11	.07
8	" + EDTA	.10	.07
9	" + DPN	.54	.42
10	" + " + EDTA	1.32	.53
11	" with EDTA, then succinate added	.45	.17

The nature of the labile substance required for the action of EDTA is suggested from the results of a typical experiment shown in Table III. It can be seen that although ATP, EDTA and DPN are very effective individually in increasing keto acid accumulation in fresh enzyme preparations, only DPN will cause such increases in aged preparations (line 9). EDTA, when added to aged preparations, is unable to stimulate keto acid production (line 8) unless DPN is added along with it (line 10), indicating that the destruction of DPN during preincubation is a factor necessary for this effect of EDTA. It should also be noted, however, that if EDTA is present during the preincubation period it protects this capacity of the tissues (line 11) probably by preserving endogenous DPN. Mitochondria contain little DPNase activity (14), a factor that may be responsible for the differences in response between preincubated mitochondria and preincubated whole homogenates to added DPN (Table III).

Comparing the effects of EDTA, DPN, and ATP on keto acid accumulation during succinate oxidation, with and without the addition of metal ions, reveals a number of striking differences: First, it was shown above that the addition of Mg^{++} completely abolishes the stimulating effect of EDTA on keto acid accumulation. Standing in strong contrast is the effect of this metal ion in *increasing* the amount of keto acids produced by DPN. However, unexpectedly this increase in keto acids with DPN and Mg^{++} is accompanied by less, or no change in the inhibition of succinate oxidation. For instance, in liver Mg^{++} causes a 4-fold increase in keto acids without affecting oxygen consumption (Table I). In this tissue these differences in keto acids are significant at the 1% level. In the heart it increases the keto acid concentration from .65 to .87 micromol per mg of nitrogen, (not quite at the 5% level) but at the same time, the oxygen consumption increases from 110 to 150 μl/mg N/hr (significant at better than 5%). Results with kidney or brain homogenates, when carried out in Warburg vessels, are more variable as far as keto acid accumulation is concerned, but in all instances with kidney the addition of Mg^{++}, along with DPN, results in a significantly (1% level) higher succinoxidase activity.[‡] However, when experiments are made in a Dubnoff shaker (no alkali used to absorb CO_2), the results show quite clearly that Mg^{++} significantly (1% level) augments the keto acid accumulation produced by DPN (Table II, column c, lines 1 and 6).

It should be pointed out that in the EDTA experiments the concentration of Mg^{++} is in excess of what would be complexed by the chelating agent(16), thus, free Mg^{++} is available. It would be reasonable to expect that if the stimulating action of EDTA on keto acid accumulation were accounted for by its ability to preserve endogenous DPN from destruction, the action of an excess of Mg^{++} should be similar with EDTA as with DPN. The differing effects suggest, then, that EDTA also affects systems that do not involve DPN or its destruction and which strongly influence the amount of keto acids accumulating during succinate oxidation.

[‡] Preliminary experiments, using paper chromatographic separation and quantitative analysis of the individual keto acids(15), indicate that sufficient OAA is present in the Mg^{++} experiments to produce considerably more inhibition of succinate oxidation than was actually observed. The averages of 2 experiments with kidney were as follows: DPN alone inhibited succinate oxidation by 55%; added together with Mg^{++}, the inhibition was 17%; of the 65 μg of total keto acids found without Mg^{++}, 45 μg were in the OAA spot. Of the 90 μg of keto acids found with Mg^{++}, 75 μg were OAA. Apparently the presence of Mg^{++} reduces ability of OAA to inhibit succinate oxidation. This phenomenon will be dealt with in detail in a subsequent paper.

ATP also stimulates keto acid accumulation during succinate oxidation (Table I, column d) but, unlike those produced by DPN, the keto acids produced by ATP do not depress succinate oxidation despite the fact that results of two preliminary paper chromatographic studies indicate the presence of sufficient OAA to produce profound inhibition. As with DPN, Mg^{++} increases keto acid accumulation produced by ATP. As for keto acid accumulation, only Ca^{++} produces similar effects on EDTA, DPN and ATP. This ion neutralizes the stimulating action of all 3. Furthermore, this inhibitory action of Ca^{++} is not antagonized by Mg^{++}.

B. *Utilization of added OAA.* Considerable variation occurs from animal to animal in the rate of utilization of OAA by the individual tissues but the relative differences in rates among the four tissues studied are very constant. The average utilization rates (μM/mg N/hr) from 14 experiments, using a Tris buffer, are: Heart, 8.20; liver, 4.20; kidney, 3.40; brain, 1.00. With phosphate buffer, the utilization rate is 25% lower. These differences are significant at the 1% level. EDTA effectively stimulates utilization, the liver usually showing the greatest response. As have others(17), we find that DPN and ATP also enhance utilization, the latter being more effective (Table II, column g). Mg^{++} or Mn^{++} is very effective in stimulating OAA utilization, tripling the rate in some instances. However, it should be noted that the system is quite active without the added ion (Table II, line 1).

In low to moderate concentrations (.0003 M to .0006 M), Ca^{++} significantly inhibits OAA utilization (Table II, column e, lines 2 and 3). Such concentrations also abolish the stimulating effect of DPN or ATP on utilization (columns f and g, line 3). All these inhibitory actions of low concentrations of Ca^{++} are readily antagonized by Mg^{++} (lines 8 and 9). This is in contrast to the irreversible Ca^{++} inhibition of keto acid accumulation. However, low concentrations of Ca^{++}, which cause approximately 50% inhibition of OAA utilization in the controls, DPN or ATP experiments, *enhance* the stimulating action of EDTA by 15 to 30% (column h, lines 2 and 3). This effect with low concentrations of Ca^{++} plus EDTA is seen with phosphate, Tris or glycylglycine buffered systems. The effectiveness of this combination can be gauged by comparing the increase with that produced by Mg^{++} (.003 M) plus EDTA, particularly in a Tris buffered medium (column j, lines 3 and 6). Ca^{++} in concentrations in excess of what will be chelated by EDTA(16), causes the expected inhibition (columns h and j, lines 4 and 5). Thus it appears that, provided no excess of this metal ion is present, a Ca^{++}-EDTA complex has a direct activating effect on OAA utilization.§

Discussion. EDTA, it appears, has a dual effect on keto acid metabolism; it stimulates the formation of OAA during succinate oxidation as well as accelerates its removal. As this keto acid is a potent inhibitor of the succinoxidase system, the overall effect on succinate oxidation depends, to a great extent, on the balance of these two activities. In all experimental conditions studied here, EDTA inhibits succinate oxidation only when it is capable of simultaneously increasing keto acid accumulation. The mechanisms that are involved in formation and utilization of OAA are not equally stimulated by EDTA as each has rate limiting factors that determine the final effect produced. For instance, it was shown that EDTA requires for its ability to stimulate keto acid accumulation the presence of a substance, apparently DPN, which is easily destroyed or depleted by preincubating the tissue at 38°C. Also it is apparent from the opposite effects on keto acid accumulation produced by added Mg^{++} in a DPN and in an EDTA medium that other systems are involved in the EDTA effect. These are primarily concerned with utilization of OAA and appear to be protected by EDTA, but are activated only when there is an excess of Mg^{++} present. Probably involved here is an ATPase as it has been shown that it is ATP that is required for the utilization of OAA (17). Hence, when both EDTA and an excess of Mg^{++} are present, the utilization of OAA exceeds production and so the keto acid does not accumulate to inhibit the succinoxidase system. The mechanism of action of Mg^{++} in a medium containing added DPN is not clear. Here Mg^{++} stimulates the accumulation of OAA yet this keto acid, under such conditions, has a diminished capacity to inhibit succinate oxidation.

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§ A phosphate buffer-dependent "diphasic" action of Ca^{++} can be observed (a reversal of the inhibitory action as concentration is increased). This, we believe, may result in misleading interpretations. Here the reversal occurs at Ca^{++} concentrations of .003 M (Table I, lines 3, 4 and 5.). With EDTA it occurs at higher concentration. This effect is not observed if Tris or glycylglycine are used as buffers.

EDTA also has a direct action in stimulating the utilization of OAA. This is seen under conditions where this agent chelates with a small amount of Ca^{++} leaving no excess of the metal ion in the medium. In all the other experimental conditions studied, Ca^{++} in low and moderate concentrations inhibits, irreversibly, formation and, reversibly, the utilization of OAA. The inhibition on the latter is readily antagonized by an excess of Mg^{++} and the mechanism can be best understood by assuming a competition between Mg^{++} and Ca^{++} for some active site on an enzyme or enzyme substrate complex. From this it can be expected that EDTA can also stimulate utilization of OAA by upsetting the Mg^{++}-Ca^{++} ratio in the cell. This possibility is buttressed by an examination of the ability of EDTA to form complexes with various metal ions(16). The ease with which Ca^{++}, or Mg^{++}, complexes with EDTA is indicated by their stability constants. Log K_2 for Ca^{++} is 10.59, for Mg^{++}, 8.69(16). This means that in a mixture of these ions EDTA tends to bind more Ca^{++}, as this complex dissociates less readily than one with Mg^{++}; or, if an Mg^{++}-EDTA complex is already formed, Ca^{++} may be expected to displace the Mg^{++} (a mechanism that may also account for the stimulating effect of low Ca^{++}-EDTA). The final result would be an increase in the effective Mg^{++} concentration which, from these results, could be expected to increase OAA utilization.

Finally, the relationships between OAA metabolism and the level of succinate oxidation demonstrated here lend further support to the hypothesis that the naturally formed OAA may have an important regulatory role on oxidative metabolism through the Krebs cycle(18).

Summary. 1. EDTA stimulates the accumulation of OAA during succinate oxidation. Although apparently DPN is required for this action, the results indicate that other systems concerned with the utilization of OAA are also involved in the EDTA effect. 2. Mg^{++}, Mn^{++}, or Ca^{++} prevent the effect of EDTA in increasing accumulation; the former 2 ions by activating a system concerned with utilization, the latter ion by inhibiting the formation of OAA. 3. Succinate oxidation is inhibited by EDTA only when the latter simultaneously stimulates OAA accumulation. 4. Mg^{++} increases OAA accumulation produced by DPN but in the presence of this ion the keto acid shows a diminished capacity to inhibit succinate oxidation.

I am indebted to Mrs. Matilda Tyler, Armonia Artes de Stiehl and Miss Graciela Coll Camalez for excellent technical assistance.

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Received January 3, 1955. P.S.E.B.M., 1955, v88.

Effects of Lecithin, Cholesterol, and Serum on Erythrocyte Modification and Antibody Neutralization by Enterobacterial Lipopolysaccharides.*

(21582)

ERWIN NETER, OTTO WESTPHAL, AND OTTO LÜDERITZ.

From Departments of Pediatrics and Statler Research Laboratories, Children's Hospital, Departments of Pediatrics and Bacteriology of Univesity of Buffalo School of Medicine, Buffalo, N. Y. and Dr. A. Wander-Forschungsinstitut, Säckingen, Germany.

Certain bacterial antigens are readily adsorbed by red blood cells and render these modified erythrocytes specifically agglutinable by homologous bacterial antibodies. In experiments with crude *Escherichia coli* antigens it was shown(1,2) that normal serum, lecithin, and cholesterol inhibit this hemagglutination reaction. Similar observations on the inhibitory effects of lecithin and cephalin on agglutination of red blood cells modified by a tuberculin fraction were subsequently reported by Boyden and Grabar(3). Inhibition may be due to either prevention of adsorption of antigen by erythrocytes or alteration of the antigen in such a way as to preclude its reaction with homologous antibody. Since the above mentioned experiments were carried out with crude antigens, it was deemed of interest to determine whether normal serum, lecithin, and cholesterol likewise inhibit hemagglutination when purified bacterial lipopolysaccharides are used. The effect of these inhibitors on antibody-neutralizing ability of antigens was also investigated.

Material and methods. Two purified enterobacterial lipopolysaccharides, obtained from *E. coli* serogroup 08 and *Salmonella abortus equi,* respectively, were prepared according to methods of Westphal and coworkers(4-6). The lipopolysaccharides were either heated at 100°C or treated with sodium hydroxide as described by Macpherson *et al.* (7), since it was shown that these procedures result in marked enhancement of erythrocyte-modifying ability without corresponding increase in antibody-neutralizing capacity(8). Lecithin (*ex ovo,* pure)[†] and cholesterol[‡] were dissolved in absolute alcohol as a 1% stock solution; appropriate dilutions were prepared daily, and corresponding alcohol dilutions employed as controls. Suitable amounts of lipopolysaccharides were mixed with lecithin, cholesterol, normal serum, or alcohol and kept either at 37°C or in boiling water for time periods specified below. These mixtures were then used for modification (10 minutes at 37°C) of either sheep or human (blood group 0) red blood cells, according to methods described previously(9-11). Serial dilutions of homologous *E. coli* and *S. abortus equi* (rabbit) antisera (vol. 0.2 ml) were mixed with an equal volume of the modified red blood cells, incubated at 37°C for 30 minutes, and centrifuged at 1500 r.p.m. for one minute; the resulting hemagglutination was noted. In the hemolysis tests, aliquots of

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[*] Aided by research grant from the National Microbiological Institute, Public Health Service.

[†] Pfanstiehl Chemical Co., Waukegan, Ill.

[‡] Fisher Scientific Co., Pittsburgh, Pa.

modified red blood cell suspensions were mixed with antiserum in serial dilutions and guinea pig complement§ in a dilution of 1:20; the mixtures were incubated at 37°C; the resulting hemolysis was recorded after incubation for 30 minutes at 37°C. The effects of above inhibitors on antibody-neutralizing capacity of lipopolysaccharides were determined by the bacterial hemagglutination or hemolysis inhibition tests as follows. Serial dilutions of untreated and inhibitor-treated antigens (vol. 0.1 ml) were mixed with 3 to 4 minimal hemagglutinating or hemolyzing units of the homologous antiserum (vol. 0.2 ml). The mixtures were kept in water bath at 37°C for 30 minutes. Red blood cells modified with 3 to 4 minimal units of homologous antigen (vol. 0.2 ml) and, in hemolysis inhibition tests, complement were added. The mixtures were incubated and results read as described above. As diluent phosphate buffer‖ (12) was used throughout.

Results. In a representative experiment 5 μg/ml of *E. coli* and 2.5 μg/ml of *S. abortus equi* lipopolysaccharides were mixed with (1) lecithin (5 mg%), (2) cholesterol (5 mg%), (3) normal sheep serum (1:200 dilution), (4) phosphate buffer, and (5) alcohol (in a concentration identical with that of lecithin and cholesterol solutions). Mixtures 1, 3, 4, and 5 were kept for 10 minutes at 37°C; mixture 2 and aliquots of 4 and 5 were heated in boiling water for 10 minutes. These materials were then used for modification of sheep red blood cells, and the modified erythrocytes tested with homologous and heterologous antisera for hemagglutination. In addition, the mixtures were used in the hemagglutination-inhibition test, utilizing both homologous and heterologous antisera and red blood cells modified with respective bacterial antigen. The results are summarized in Table I.

TABLE I. Effects of Lecithin, Cholesterol, and Serum on Erythrocyte-Modifying and Antibody-Neutralizing Capacities of Enterobacterial Lipopolysaccharides.

	A Hemagglutination* by		B Neutralization* of	
Antigen*	*E. coli* 08 antisera	*S. abortus equi* antisera	*E. coli* 08 antisera	*S. abortus equi* antisera
E. coli 08 lipopolysaccharide	+	—	+	—
Idem & lecithin	—	—	+	—
" cholesterol	—	—	+	—
" serum	—	—	+	—
" alcohol	+	—	+	—
S. abortus equi lipopolysaccharide	—	+	—	+
Idem & lecithin	—	—	—	+
" cholesterol	—	—	—	+
" serum	—	—	—	+
" alcohol	—	+	—	+

* For details see text.

+ = Hemagglutination in modification test (A) or antibody-neutralization in hemagglutination inhibition test (B).

— = No hemagglutination in modification test (A) or no antibody-neutralization in hemagglutination inhibition test (B).

This Table reveals the following. (1) Lecithin, cholesterol, and normal sheep serum inhibited homologous bacterial hemagglutination. (2) These materials did not interfere with antibody-neutralizing capacity of antigens. (3) This antibody-neutralization is specific, since neither antigens alone nor mixtures of antigen and inhibitors were capable of inhibiting hemagglutination in the heterologous system. These results indicate that the inhibitory action on hemagglutination is not due to an alteration of lipopolysaccharide antigens which precludes their reactivity with the homologous bacterial antibodies. Rather, the inhibitors apparently interfere with adsorption of lipopolysaccharides by red blood cells.

Essentially identical results were obtained in hemolysis and hemolysis inhibition experiments. Further studies revealed that pretreatment of red blood cells with lecithin prior to modification with lipopolysaccharides also resulted in inhibition of hemagglutination and hemolysis. In contrast, pretreatment of erythrocytes with cholesterol and normal serum had no such effect. It is interesting to mention that heated cholesterol solution (0.5 mg%) did not inhibit erythrocyte-modifying capacity of lipopolysaccharides, whereas, as shown above, heating of cholesterol-antigen mixtures resulted in failure of erythrocyte-modification. These findings strongly suggest that an interaction takes place during heating between lipopolysaccharide and cholesterol. So far as normal serum is concerned, it was found that sera from man, sheep, and calf had inhibitory effects, and that the sera were effective when red blood cells from the same animal species and even from the same individual were used in the hemagglutination test.

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§ Carworth Farms, New City, N. Y.

‖ Difco Laboratories, Detroit, Mich.

Discussion. The present study with 2 purified enterobacterial lipopolysaccharides confirms the results of previously reported experiments with crude *E. coli* antigens to the effect that lecithin, cholesterol, and normal serum inhibit hemagglutination by homologous bacterial antibodies. Since no evidence was obtained that either material destroyed the antibody-combining capacity of antigens, it is apparent that the inhibitory effect is due to interference with adsorption of antigen by erythrocytes. Boyden and Grabar(3) found that extracts of red blood cell stromata as well as lecithin and cephalin and, to a lesser extent, cholesterol, inhibited agglutination of red blood cells modified by a tuberculin extract. It is conceivable that cholesterol may have been more effective, had the tuberculin antigen been heated with the inhibitor as in the present experiments. It is worthy of note that, according to Morimoto and Morgan (13), lecithin, cholesterol, and cardiolipin reduce activity of mumps virus hemolysin. Similar to the above experiment with enterobacterial lipopolysaccharides, pretreatment of erythrocytes with lecithin, but not with cholesterol, rendered them less susceptible to lysis by mumps virus. Thus, lecithin acts as an inhibitor of mumps virus hemolysis and enterobacterial hemagglutination after reaction with either antigen or erythrocyte. Further studies are needed to determine mode of action of inhibitors of enterobacterial hemagglutination and to elucidate the nature of erythrocyte receptors. Studies on cell receptors for bacterial antigens are of particular interest, since certain bacterial polysaccharide products inhibit virus hemagglutination (Macpherson *et al.*(7)) and lessen the severity of infection with pneumonia virus of mice (Horsfall and McCarty(14)).

Finally, since the enterobacterial lipopolysaccharides are known pyrogens and normal serum contains substances which augment and probably also inhibit pyrogenicity(15), the possible effects of lecithin, cholesterol, and plasma fractions on fever-producing capacities of these antigens deserves further exploration.

Summary. 1. Sheep red blood cells treated with mixtures of purified enterobacterial (*E. coli* 08 or *S. abortus equi*) lipopolysaccharides and lecithin, cholesterol, or normal serum were not agglutinated by homologous bacterial antibodies. 2. Pretreatment of erythrocytes with lecithin but not with cholesterol or compatible normal serum had similar effects. 3. The antibody-neutralizing capacity of the lipopolysaccharide-inhibitor mixtures was identical with that of the lipopolysaccharides, as revealed by hemagglutination inhibition tests. 4. It is concluded that the inhibitors interfere with adsorption of enterobacterial lipopolysaccharides by erythrocytes.

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Received January 13, 1955. P.S.E.B.M., 1955, v88.

Plasma Glutamine and Oxypurine Content in Patients with Gout. (21583)

STANTON SEGAL AND JAMES B. WYNGAARDEN. (Introduced by R. W. Bates.)

From National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Department of Health, Education, and Welfare, Bethesda, Md.

A relationship between glutamine and purine synthesis was suggested some years ago by Orström, Orström, and Krebs(1), who observed that the rate of hypoxanthine synthesis by pigeon liver slices was accelerated when oxalacetate and glutamine were added to an incubation mixture which supplied nitrogen as ammonium chloride. On the basis of subsequent studies, Orström *et al.*(2) suggested that glutamine might participate in hypoxanthine synthesis as a nitrogen carrier. Recently, Sonne, Lin, and Buchanan(3) have clearly established the participation of the amide nitrogen of glutamine in the biosynthesis of the purine ring. Somewhat prior to this latter work, Orström and Orström(4) investigated blood glutamine and oxypurine levels in gouty subjects, and reported that glutamine levels were remarkably low and oxypurine levels decidedly high in this disease. A decrease of blood glutamine to zero was reported to occur in one subject during acute gouty attacks. A stoichiometric relationship between amount of glutamine disappearing and amount of oxypurine appearing in blood was described in cases of gout, and a novel chemical mechanism for conversion of a portion of the glutamine molecule to the purine ring was proposed.

In the present study glutamine and oxypurine levels have been measured in the plasma of 10 gouty and 10 control subjects. Two gouty subjects were studied during the latter days of an acute attack; 8 were studied during an intercritical phase of the disease. This paper reports an inability to confirm the observations of Orström and Orström.

Methods. Venous blood was collected and plasma separated with precautions as suggested by Seegmiller, Schwartz, and Davidson(5). *Plasma glutamine* content was determined by 2 methods on the same plasma samples, utilizing both the specific glutaminase of *E. coli*, strain W,* and also an acid hydrolysis method. Glutaminase was prepared according to Meister(6) and purified through the stage of lyophilization of the 0.1 M KH_2PO_4 extract of calcium phosphate gel. 1.25 mg of this powder dissolved in 0.25 ml of 0.1 N acetate buffer pH 4.9 was sufficient to liberate 20 μg of glutamine amide-N during one-half hour incubation at 37°, and was inactive when asparagine or adenosine was substrate. For enzymatic assay, one ml of plasma previously adjusted to pH 4.9 with 4N acetic acid was incubated with 0.25 ml of enzyme solution plus 0.1N acetate buffer pH 4.9 in a final volume of 1.5 ml. The mixtures were prepared in 30 ml penicillin bottles† and incubated at 37° C in a Dubnoff metabolic shaker. After 30 minutes, ammonia was liberated by the addition of 1 ml of saturated potassium carbonate. The acid hydrolysis method was modified after Krebs(7), using a 7.5% trichloroacetic acid filtrate and 5% sulfuric acid (1 ml filtrate and 0.1 ml 50% sulfuric acid). After the 10-minute hydrolysis period, 0.2 ml of saturated NaOH and 1 ml of saturated potassium carbonate were added. Standard glutamine and ammonium sulfate solution, reagent blanks, and recoveries of added glutamine were determined with each set of analyses for both methods. The liberated ammonia was determined by a modification of the microdiffusion method of Seligson and Seligson(8). Ammonia was collected on the tip of a ground glass rod, which had been dipped in 2 N H_2SO_4. Microdiffusion was facilitated by agitation of the bottles for 30 minutes on their sides in a Kahn shaker, oscillating 144 times a minute. The rods were then removed and immersed in 10 ml of water to which had been added 1 ml of Nessler's solution prepared according to Vanselow(9). After 15 minutes the solutions were transferred to cuvettes and optical density determined at 420 mμ in a Coleman Jr. Spectrophotometer. All determinations were done in duplicate. By the enzymatic method, recovery of 10 μg of glutamine amide-N added to 1 ml of normal or gouty plasma ranged from 92 to 104%. By the acid hydrolysis method, recovery of 10 μg of glutamine amide-N added to plasma filtrates was 81 to 118%. *Uric acid* was determined according to Praetorius' modification(10) of the differential spectrophotometric method of Kalckar(11) employing purified hog liver uricase prepared according to Müller and Bauer (12). Oxypurines were estimated on the same samples of sera by determining the increment in uric acid content following incubation with highly purified xanthine oxidase. One ml of serum and 2.5 ml of 0.5 M glycine buffer pH 8.2 were diluted to 25 ml. Three ml of this mixture were then transferred to a quartz cuvette, and placed in the Beckman Model DU Spectrophotometer, containing a "gray filter" reference cell consisting of a solution of uric acid of approximately 15 μg/ml. as the lithium salt. 0.01 ml of xanthine oxidase solution was added to the experimental cell and the optical density at 292 mμ recorded until no further change occurred for a period of 5 minutes. 0.01 ml of uricase suspension was then added and the uric acid content again determined. The oxypurine content, expressed as hypoxanthine, was then calculated as follows:[‡]

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* The authors are indebted to Dr. Alton Meister for a generous gift of *E. coli,* strain W.

† No-Sol-Vit glass vials, Type I USP glass, 20 mm opening.

$$\frac{-\Delta \overset{u + xo}{OD}_{292\ m\mu} - (-\Delta \overset{u}{OD}_{292\ m\mu})}{-.0725} \times \frac{136}{168} \times 25 =$$

mg hypoxanthine per 100 ml serum. Xanthine oxidase was prepared[§] according to Horecker and Heppel(13) through the stage of ammonium sulfate fractionation using warm milk as starting material, as suggested by Kalckar, Kjeldgaard, and Klenow(14).

Results. The plasma glutamine values are presented in Table I. By the enzymatic method the normal range is 7.0 to 10.1 mg % with a mean of 8.9. By the acid hydrolysis method (same plasma specimens) the normal range is 6.2 to 11.8 mg % with a mean of 9.5. In the patients with gout, the plasma glutamine content as determined by the glutaminase method ranged from 6.8 to 9.8 mg %, with a mean of 8.4, and as determined by the acid hydrolysis method ranged from 7.0 to 10.9 with a mean of 8.6. Satisfactory agreement is apparent between the ranges and means for both methods within the 2 subject groups although there was imperfect correlation between results of the two methods carried out on the same plasma sample. It is clear that by these 2 technics there was no difference between the values for plasma glutamine content in control and gouty subjects. The normal values herein reported are in agreement with those previously obtained with a variety of technics(4,15-19), although somewhat higher than those recorded by Krebs(20).

The "plasma blanks" apparently do not indicate plasma ammonia content. Several investigators have pointed out that there is no true ammonia in fresh normal plasma, the apparent ammonia reflecting ammonia released from constituents of blood on standing (21,22). The ranges and mean values of plasma blanks expressed as NH_3-N were not significantly different in the control and gouty subjects, although the two technics gave different values (see Table I). The high blanks by the acid hydrolysis method probably reflect the increased liberation of NH_3 in the trichloroacetic acid media. The plasma blanks obtained by the enzymatic method in both control and gouty subjects are in complete agreement with normal values reported by Seegmiller, Schwartz, and Davidson(6).

Table I also presents the serum uric acid and oxypurine levels. In the control subjects, the uric acid levels ranged from 3.5 to 5.9 mg %, with a mean of 5.1; in the gouty subjects the uric acid levels ranged from 6.8 to 10.8%, except for one value of 4.4 mg % in a patient receiving probenecid.

Plasma oxypurine values ranged from 0 to

[‡] u = uricase, xo = xanthine oxidase. Determination of the relative amounts of hypoxanthine and xanthine present, by measurements of change of O.D. at 244 mμ, the isosbestic point of xanthine and uric acid, were found to be unreliable when the molar ratio of total oxypurine to uric acid was less than 1:4.

[§] The authors are indebted to Dr. J. E. Seegmiller for a generous gift of xanthine oxidase.

TABLE I. Plasma Glutamine, Uric Acid, and Oxypurine Levels.

	Glutaminase hydrolysis		Acid hydrolysis			
Subject, age, clinical status	NH_3-N, µg/ml plasma	Glutamine, mg %	NH_3-N, µg/ml plasma	Glutamine, mg %	Uric acid, mg %	Oxypurine, mg %
I. Controls						
♂, 21 yr	1.5	7.0			5.0	.09
♂, 27	1.2	9.0				
♂, 30	1.6	9.3			5.8	.25
♀, 53	.9	9.7			4.6	.22
♀, 28	1.1	8.9	5.9	6.2	3.5	.23
♂, 28	1.0	10.1	6.4	9.5	4.8	
♂, 27	1.8	7.7	6.9	11.3	5.9	.00
♂, 32	.7	9.6	6.7	11.8	5.3	.00
♂, 23	1.0	8.4	2.6	8.4		
♂, 29	.9	9.6			5.8	.14
Range	.7–1.8	7.0–10.1	2.6–6.9	6.2–11.8	3.5–5.9	.00–.25
Avg	1.2	8.9	5.7	9.5	5.1	.13
II. Gout (all ♂)						
48, 10 yr i, t, c	1.5	9.7			8.1	.28
52, 16 yr i, t, p	.8	9.8	5.7	7.0	6.9	.03
54, 20 yr i, no t, no Rx	1.1	8.2	4.9	8.4	6.8	.17
49, 4 mo i, no t, pr	2.2	7.8	4.4	10.9	6.9*	
62, 12 yr i, t, c	1.0	7.9	5.6	7.0	8.0	.25
51, 16 yr i, t, c	1.0	8.3	2.3	9.6	10.8	.17
50, 5 yr i, no t, no Rx	1.1	9.6			8.8	.20
52, 10 yr, 1 wk after acute attack; no t, pr, c	1.4	7.9			4.4*	.42
53, 16 yr acute attack, t, no Rx	2.0	6.8			7.5	.17
51, 3 yr acute attack, no t, no Rx	1.0	7.5			9.7	.11
Range	.8–2.2	6.8–9.8	2.3–5.7	7.0–10.9	6.8–10.8*	.03–.42
Avg	1.3	8.4	4.6	8.6	8.3*	.19

* Omitting subjects who were taking probenecid.
i = intercritical; t = tophi; c = colchicine; p = phenylbutazone; pr = probenecid.

.25 mg % in 7 control subjects with a mean of .13, and from .03 to .42 mg % in 9 gouty subjects with a mean of .19 mg %. No apparent correlation was found between glutamine, oxypurine, and uric acid levels in either group.

Discussion. Using an acid hydrolysis method for determining blood glutamine levels, Orström and Orström(4) found values in 15 control subjects ranging from 7.4 to 14.1 mg %, with a mean of 9.95 mg %. These values are comparable to those reported for control subjects in the present study, as determined both by acid hydrolysis and glutaminase methods. The same investigators reported blood glutamine levels in 8 gouty subjects, 2 of whom were in an acute attack, ranging from 0 to 3.1 mg %, with a mean of 1.4 mg %. In the present study, the plasma glutamine levels in 10 gouty subjects representing various stages of the disease were normal by both analytical methods employed.

In the normal subjects studied by Orström and Orström, oxypurine levels were observed ranging from 0 to 1.5 mg %, with a mean of .4 mg % (expressed as hypoxanthine). In their gouty patients, levels of 8.3 to 15.0 mg % were recorded, with a mean of 10.5 mg %. The method employed was not highly specific, depending on a measurement of oxygen consumption when xanthine oxidase was added to blood. Wolfson and Cohn(23) found a range of oxypurine levels of 0.2 to 0.6 mg % in normal subjects, employing a combination of xanthine oxidase incubation and a colorimetric estimation of the increment of urate thus produced. In 7 of 9 gouty subjects, levels of 0.0 mg % were recorded at least once, but 7 of this group also had elevated oxypurine levels on at least one occasion. Values of 0.8 to 3.8 mg % followed adrenocorticotropin administration in 6, and one of 3.8 mg % was noted shortly after recovery from an acute attack in the remaining subject.

In the control subjects of the present study oxypurine levels ranged from 0 to 0.25 mg %. Results in the gouty subjects were within this range in 7 instances, and greater than 0.25 mg % in only 2 cases. The highest value, 0.42 mg %, was found in a patient 3 days after cessation of acute gout. Although the highly specific oxypurine method employed has yielded values lower than those of Wolfson and Cohn, the results are in general agreement with their findings that patients with gout may on occasion show increased serum oxypurine levels. Neither their results nor ours are in agreement with those of Orström and Orström regarding the magnitude of these increases.

Overproduction of uric acid has been demonstrated in certain gouty subjects by means of studies of the rate of incorporation of labeled glycine into uric acid(24). It is possible that an overproducer of uric acid would also show a low serum glutamine level due to increased utilization of glutamine in purine synthesis, and a high serum oxypurine level. However, Subject 7, who excreted 822 mg of uric acid per day while on a low purine diet, had normal levels of both glutamine and oxypurine in plasma.

Summary. Plasma glutamine and oxypurine levels were studied in a group of 10 control and 10 gouty subjects. No differences could be observed in the plasma glutamine content between the 2 groups. Plasma oxypurine level was clearly elevated in only one gouty subject. This study fails to confirm a previous report of abnormally low levels of glutamine and markedly high levels of oxypurines in blood of patients with gout.

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Received January 24, 1955. P.S.E.B.M., 1955, v88.

Immediate Uricosuric Effect of Probenecid in Normal Humans. (21584)

Charles Bishop and William Pfaff. (Introduced by Fred R. Griffith, Jr.)

From Chronic Disease Research Institute, University of Buffalo Medical School, Buffalo, N. Y.

In an earlier study from this Institute it was shown that the drugs, probenecid (Benemid),[*] and phenylbutazone (Butazolidin),[†] exerted uricosuric action only in patients with gout, and not in patients with rheumatoid arthritis(1). A later study using N^{15} uric acid(2) demonstrated that both drugs were effective in decreasing the uric acid pool size, increasing the uric acid turnover rate, and decreasing the serum urate level in patients with rheumatoid arthritis whose body "stores" of urate were within normal limits. Similar but less striking effects were noted in gouty patients and patients with rheumatoid arthritis who had an elevated serum urate level and an increased uric acid pool size. Since the subjects with normal urate stores appeared to lose urate as judged by their pool sizes and serum urate levels, and since this "extra" urate did not appear in the urine, it became of interest to know what happened to it. One must now recall that the test periods for the previous two studies were of one to two weeks duration, the means of such periods being compared, using the t test to point out significant differences. It was apparent that the experimental design of the previous studies would demonstrate more permanent drug effects but might tend to conceal transient effects. An experimental design was then evolved which would show what happened to uric acid excretion in the first few hours and the first 2 days after probenecid administration. The results show that in normal individuals there is a prompt outpouring of "extra" uric acid into the urine and that the quantity excreted within the first day may easily account for the amount lost from the body pool and serum urate. Presumably after that the rate of excretion approaches normal again since equilibrium of production and disposal must again obtain. In this way no long term uricosuric effect will be noted.

Methods. Two healthy normal male laboratory workers in the 3rd and 4th decades were the experimental subjects. In order to minimize variations of uric acid excretion caused by the ingestion of food, a standard regimen was ingested each hour beginning at the hour of arising and continuing throughout the day. Supper was allowed *ad libitum*. The hourly feeding consisted of 2 crackers, 7.5 g cheese, 2 small sugar wafers, and 80 ml homogenized fresh milk, the total being equivalent to 150 calories. The subjects voided just before each feeding and drank extra water to insure a sufficient urine volume (preferably about 100 ml/hr). The uric acid concentrations of each urine sample were determined by the enzymatic spectrophotometric method of Praetorius(3). The first 1

TABLE I. Rate of Urinary Uric Acid Excretion (mg/min.) before and after Ingestion of Probenecid in Normal Male (W.P.).

	Control		Probenecid	
Period	Day 1, mg/min.	Day 2, mg/min.	Day 3,[†] mg/min.	Day 4,[†] mg/min.
1[*]	.521[*]	.444	(.482)[‡]	.860
2	.560	.517	1.297	1.121
3	.555	.508	1.794	.795
4	.525	.476	1.612	.790
5	—	.436	1.345	
6	.407		1.182	
7	.365		1.238	
Mean	.489	.476	1.408	.892
Stand. dev.	.082	.037	.242	.156
Combined mean	.483			
Combined stand. dev.	.063			

[*] On days 1 and 2, first feeding at 7 A.M., first urine collected at 10 A.M. On day 3, first feeding at 7:45 A.M., first urine collected at 10:45. On day 4, first feeding at 9 A.M., first urine collected at 11 A.M.

[†] On day 3, probenecid at 10:50 A.M., 4 and 8:30 P.M.; on day 4, at 9 A.M., 2:30 and 6 P.M.

[‡] The value must not be included in the mean since the first dose of probenecid was administered just after it.

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[*] p(di-n-propylsulfamyl) benzoic acid, kindly supplied through the courtesy of Sharp and Dohme, Philadelphia, Pa.

[†] 3,5 dioxo-1, 2 - diphenyl - 4 -n- butyl pyrazolidine, Geigy Pharmaceuticals Division of Geigy Co. Inc., New York City.

TABLE II. Rate of Urinary Uric Acid Excretion (mg/min.) before and after Ingestion of Probenecid in Normal Male (C.B.).

	Control		Probenecid	
Period	Day 1, mg/min.	Day 2, mg/min.	Day 3,† mg/min.	Day 4,† mg/min.
1*	.640*	.373	.546	.777
2	.534	.450	1.480	.725
3	.585	.394	1.580	.552
4	.538	.452	1.391	.595
5	.510	.362	1.266	
6	.472		1.288	
7	.350			
Mean	.518	.406	1.258	.662
Stand. dev.	.092	.043	.368	.106
Combined mean	.472			
Combined stand. dev.	.092			

* First feeding at 7 A.M., first urine collected at 10 A.M.

† On day 3 probenecid at 9 A.M., 12:30, and 6 P.M.; on day 4, at 7 A.M., 12, and 6 P.M.

or 2 hourly samples of each day were discarded in order to allow equilibrium to obtain. The first 2 of 4 successive days were the control days. On the third day each subject ingested 3 x 0.5 g probenecid, the first dose being taken just before (C.B.), or just after (W.P.) the first urine sample that was saved. On the fourth day the regimen of the previous day was followed but by this time the subjects had of course been on probenecid for nearly 24 hours and the object was to observe the continued effect of the drug.

Results. The rates of excretion of urinary uric acid for all the periods are shown in Tables I and II. It is obvious that probenecid causes a prompt increase in urate excretion when it is taken the first day. Its effect in the second day is less marked. To quantitate this effect, t tests(4) were performed on the combined control periods (Days 1 + 2) as against Day 3 or 4. Day 3 was also tested against Day 4. The results are shown in Table III.

It seems quite apparent that probenecid causes a prompt outpouring of urinary uric acid even in the normal subject who has no unusual uric acid stores. It is known from the work of Sirota *et al.*(5) that probenecid markedly increases urate clearance in the gouty subject. In the present study urate clearances were performed in the 3rd day of probenecid administration and were found to be markedly elevated over the control clearances (control urate/inulin clearances about 10%, after probenecid, over 40%).

Discussion. In one of the previous studies (2) it was shown that probenecid caused the uric acid pool size to decrease to about half its control value in two patients with rheumatoid arthritis who had normal body stores of uric acid. On the assumption that a similar effect might occur with the two normal subjects in the present study, an arbitrary calculation was performed to see how long it would take to excrete such an amount of uric acid. If one assumes that a normal uric acid pool size for males is around 1000 mg, then to reduce this to half its size would require the excretion of 500 mg of "extra" uric acid. For Subject W.P., the mean excretion in the combined control periods was .483 mg/min. In the first day of probenecid, the mean excretion was 1.408 mg/min. If, for the sake of calculation, one assumes that the difference between the two represents the rate of excretion of "extra" uric acid, then it would take

$$\frac{500 \text{ mg}}{(1.408 - .483) \text{ mg/min.}} = 541 \text{ min. } (9.0 \text{ hr})$$

to reduce the uric acid pool size to half its value under these conditions. Similar calculations for Subject C.B. give the value of 636 min. (10.1 hr). A factor which complicates any calculations of this type is that in the normal subject only about 75% of the body uric acid turned over is excreted in the urine (6). Our experience suggests that this figure may increase after a uricosuric agent such as probenecid because the uric acid has less opportunity to be lost through competing routes of disposal.

One may tentatively conclude from the 3 papers in this series that uricosuric agents

TABLE III. Result of t Tests on Urinary Uric Acid Excretion before and after Probenecid Administration.

	p value	
Periods tested	Subject W.P.	Subject C.B.
Control *vs.* 1st pr day*	<0.5	<0.5
" " 2nd "	<0.5	<0.5
1st pr day *vs.* 2nd pr day	1>p>0.5	2.5>p>1

* pr day = probenecid day.

which presumably act by decreasing tubular resorption of urate can induce a brief uricosuria in humans with no urate stores (solid phase urate) but that this uricosuria will be self-limiting because an equilibrium between urate production and excretion must be re-established, although at a lower serum urate level. The uricosuria can persist in patients who have urate deposits to draw upon. This simplified explanation ignores more complicated shifts in equilibria.

Summary. Urinary uric acid output of 2 healthy male subjects on an hourly feeding regimen was markedly increased on the first day of probenecid ingestion. The amount of uric acid excreted the second day of probenecid administration was greater than normal but not as great as on the first day. This is in contrast to the previous finding that patients with normal amounts of body urate do not show a long term uricosuria after probenecid or phenylbutazone. It is suggested that these drugs induce uricosuria in all cases but that this uricosuria is self-limiting in subjects with no body stores of urate since equilibrium between urate production and disposal must be re-established.

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Received January 25, 1955. P.S.E.B.M., 1955, v88.

Influence of DCA and Cortisone on Adrenal Glands of Fed and Acutely Starved Hamsters. (21585)

Karl M. Knigge. (Introduced by J. C. Donaldson.)

From the Department of Anatomy, University of Pittsburgh School of Medicine, Pittsburgh, Pa.

As in many species, adrenal glands of the hamster atrophy after hypophysectomy(1) and after administration of desoxycorticosterone acetate(2), while after acute starvation(3) and exposure to cold(4) they exhibit histological evidence of increased secretory activity. In contrast to almost all other species, cortical cells in the zona fasciculata of the normal gland of the hamster contain only traces of sudanophilic lipid or cholesterol(1, 5-7). Formalin-fixed frozen sections exhibit positive tests with several aldehyde or carbonyl reagents, but the reactive material is not extracted with fat solvents. A second unique feature of the hamster adrenal is that cells of the zona glomerulosa normally contain droplets of fat believed to be a free (non-protein bound) phospholipid(1). Several aspects of adrenal physiology in the hamster, including sodium and potassium levels after adrenalectomy(8,9) and the autonomy of the zona glomerulosa(2), appear to be significantly different from those observed in other animals(9-12).

Because exogenously administered desoxycorticosterone acetate (DCA) results in compensatory adrenal atrophy, the current experiment was undertaken to determine whether DCA or cortisone acetate can produce adrenal involution in acutely starved hamsters.

Material and methods. Male hamsters, 95-105 g initial body weight, were divided into the following groups: (A) control animals, fed *ad libitum* on Purina Laboratory Chow; (B) animals deprived of all nutriment, except water, until they lost 25-30% of initial body weight; (C) fed and acutely starved hamsters given daily subcutaneous injections of varying amounts of DCA (Percorten, Ciba) or

TABLE I. Influence of DCA and Cortisone Acetate on Body and Organ Weights of Fed and Acutely Starved Male Hamsters.

Group	No. of hamsters	Body, g		Organs, mg				
		Initial	Final	Adrenals	Thymus	Spleen	Heart	Kidneys
C* ′	18	95.8 ± 3†	97.3 ± 4	24.2 ± .8	65.5 ± 5	114 ± 5	356 ± 12	789 ± 10
S*	10	105.3 ± 2	77.9 ± 2	22.4 ± .4	18.6 ± 3	84.0 ± 3	353 ± 14	748 ± 12
Fed + 2 mg DCA	8	97.5 ± 4	99.0 ± 4	17.8 ± .8	—	—	—	—
" + 5 " DCA	9	103.4 ± 2	105.2 ± 4	15.5 ± 1.1	57.2 ± 4	102 ± 2	412 ± 12	1362 ± 32
S + 5 " DCA	12	97.2 ± 3	72.3 ± 5	23.3 ± .7	25.3 ± 4	80.5 ± 3	271 ± 11	1067 ± 24
Fed + 5 " CA*	7	98.4 ± 4	87.2 ± 5	24.0 ± 1.0	20.7 ± 4	37.0 ± 2	444 ± 8	1333 ± 28
" +10 " CA	8	99.0 ± 3	87.4 ± 3	21.3 ± .9	3.5 ± .8	37.7 ± 1	448 ± 10	1281 ± 37
S + 5 " CA	10	100.5 ± 5	65.8 ± 5	23.0 ± 1.1	0	47.6 ± 3	281 ± 6	737 ± 8
S +10 " CA	10	103.1 ± 2	72.5 ± 3	22.9 ± 1.0	0	46.6 ± 2	371 ± 9	972 ± 14

* C = Control; S = Starved; CA = Cortisone acetate. † Mean ± stand. dev.

cortisone acetate (Cortone, Merck);* the hormones were administered in divided doses for the same number of days as required for untreated, starved animals to lose 25-30% of body weight (7-8 days). At autopsy, adrenals, thymus, spleen, heart and kidneys were weighed. Adrenals were prepared for histological examination by several methods, described elsewhere in detail(1,2) to demonstrate mitochondria, alkaline phosphatase and esterase enzyme activity, sudanophilic and osmiophilic lipids, cholesterol and aldehydes.

Results. Body and organ weights. Administration of 5 or 10 mg cortisone acetate daily for 7 days causes fed hamsters to lose 11% body weight, while DCA in doses up to 5 mg/day does not affect body weight (Table I). Doses of DCA greater than 5 mg/day are not well tolerated by hamsters, the animals becoming moribund and dying in 5-6 days. Adrenal weight in DCA-treated hamsters is reduced approximately 35%, while that of cortisone acetate-treated animals is not altered significantly. Body weight of hamsters acutely starved for 7-8 days declines an average of 26%, while their adrenal weight decreases only slightly. Administration of 10 mg of cortisone acetate or 5 mg of DCA during the period of starvation does not reduce adrenal weight more than occurs in untreated, starved animals. Cortisone acetate reduces strikingly the weight of thymus and spleen, an effect noted in other species also(13-14). DCA increases the weight of kidneys in both fed and starved hamsters, while cortisone acetate appears to exert notable renotropic effects only in fed animals (Table I).

Effect of acute starvation on adrenal cortex. Size of cell clusters as well as size of individual cells of the zona glomerulosa are reduced, while the number of glomerulosal lipid droplets is increased markedly compared to sections of normal adrenals. Mitochondria in cells of this zone increase in size and stain more intensely than normal (Fig. 1, 4). Alkaline phosphatase and esterase activity are not altered notably. Cells of the zona fasciculata are somewhat smaller after acute starvation, while their mitochondria are likewise increased in size. Lipid droplets, histochemically similar to those in the zona glomerulosa, are present in almost all cells of the zona fasciculata (Fig. 4).

Effect of DCA and cortisone on adrenal cortex of fed hamsters. The zona glomerulosa in adrenals of DCA-treated animals is not affected significantly, appearing normal with respect to size of its constituent cells, number and distribution of lipid droplets, and degree of phosphatase and esterase activity. Cells of the zona fasciculata are reduced in size and exhibit marked reductions in enzyme activity. In mitochondrial preparations, fasciculata cells are filled with large, unique mitochondria-like bodies (liposomes), similar to those appearing in fasciculata cells of adrenals of hypophysectomized hamsters(1).

Cortisone acetate, in doses up to 10 mg/day, produces changes in adrenal cytology significantly different from those observed after DCA. In the zona glomerulosa, the most conspicuous effect is a marked increase

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* The author wishes to thank Doctor Robert Gaunt of the Ciba Pharmaceutical Co. for the desoxycorticosterone acetate and Doctor Harry Robinson of Merck and Co. for the cortisone acetate.

FIG. 1-6. Hamster adrenal cortex, fixed in Regaud's fluid, sectioned at 4 μ and stained with iron hematoxylin. × 650.

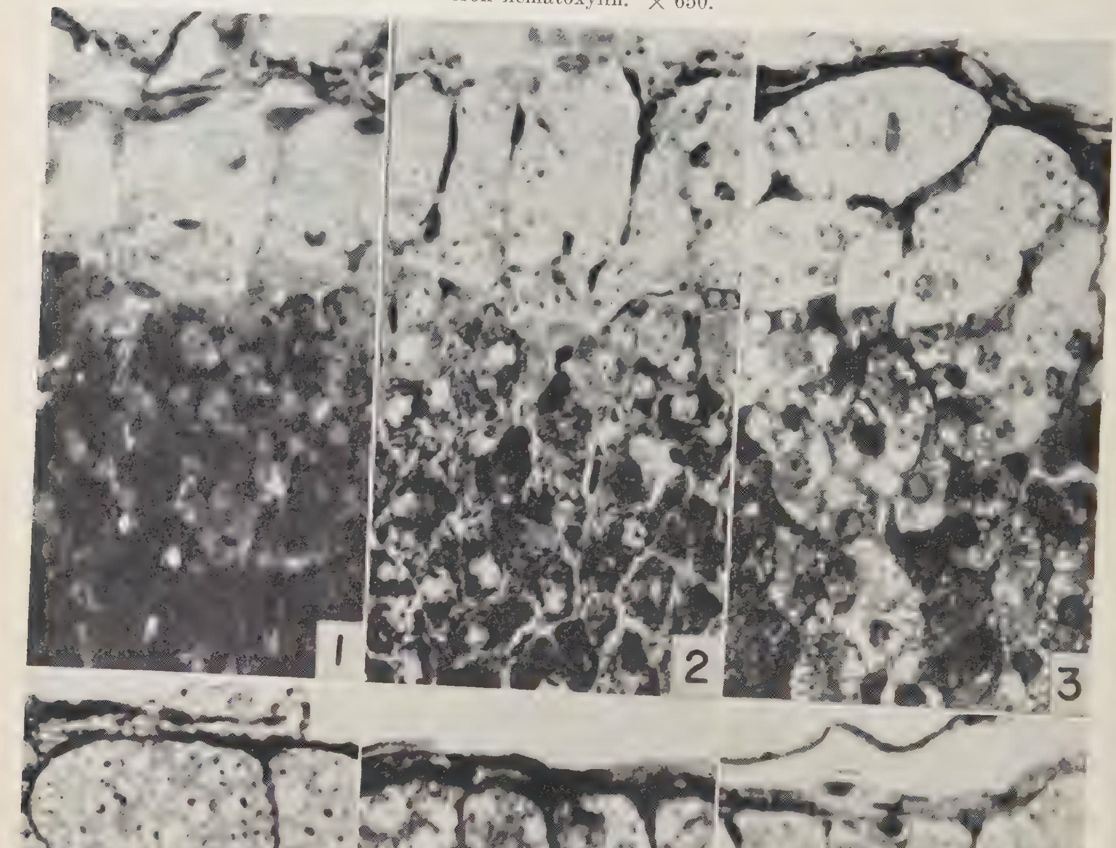

FIG. 1. Control. Because of differences in staining intensity of the mitochondria, the zona fasciculata is demarcated sharply from the zona glomerulosa.

FIG. 2. Fed + 2 mg DCA. The glomerulosa is not affected. Cells of the zona fasciculata are reduced in size and their mitochondria are altered.

FIG. 3. Fed + 10 mg cortisone. Cytological changes are similar to those produced by acute starvation (Fig. 4).

FIG. 4. Starved. Glomerulosal cells exhibit increased vacuolation and the zona stains darker because mitochondria are more prominent. Vacuoles are present in fasciculata cells and their mitochondria are larger than normal.

FIG. 5. Starved + 5 mg DCA. This hormone does not alter significantly the changes produced by starvation.

FIG. 6. Starved + 10 mg cortisone. Vacuolation of fasciculata cells appears more marked than is produced by starvation alone.

in number of lipid droplets (Fig. 3). Size of glomerulosal cells, enzyme activity, and tests for lipids are not altered notably. Cells of the zona fasciculata appear slightly smaller, their mitochondria are larger than normal, and their cytoplasm contains lipid droplets similar to those present in adrenals of starved animals.

Effect of DCA and cortisone on adrenal cortex of starved hamsters. The cytological and histochemical changes produced by acute starvation are not altered significantly by daily administration of either 5 mg of DCA or 5 mg of cortisone acetate (Fig. 5). In animals given 10 mg cortisone acetate, the number of lipid driplets in fasciculata cells is greater than that observed in adrenals of untreated, starved animals (Fig. 4, 6). Phosphatase and esterase activity appear reduced somewhat because of this marked vacuolation.

Discussion. In the fed hamster, compensatory adrenal atrophy, as judged by changes in adrenal weight and cytology, is not produced readily by administration of cortisone acetate in doses which reduce markedly lymphoid tissue and which cause loss of body weight. In contrast, DCA is effective in producing reduction in adrenal weight and cytological changes similar to those resulting from hypophysectomy.

Acute starvation is generally considered as a stressful condition in which an adrenal response is evoked leading to increased secretion of adrenocortical hormones. Cytological changes observed in adrenals of acutely starved hamsters are considered to reflect increased secretory activity. This view is strengthened by current observations that, whereas DCA readily produces adrenal involution in fed hamsters, doses of this hormone up to 5 mg/day do not lead to compensatory atrophy in starved hamsters, and furthermore, do not alter significantly the signs of hyperactivity noted in these glands.

Summary. Compensatory adrenal atrophy is produced readily in fed hamsters by DCA (2-5 mg), whereas cortisone acetate (5-10 mg) does not affect adrenal weight significantly. The cytological changes observed in adrenals of fed animals after cortisone acetate administration are similar to those found in adrenals of acutely starved hamsters. Neither DCA nor cortisone acetate alter significantly the adrenal changes produced by acute starvation.

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Received January 27, 1955. P.S.E.B.M., 1955, v88.

Induction and Inhibition of Adaptive Enzyme Formation in a Phytoflagellate.* (21586)

Vincent P. Cirillo. (Introduced by Joseph B. Goldsmith.)

From Department of Preventive Medicine and Public Health, University of Oklahoma School of Medicine, Oklahoma City.

In a previous study of the adaptive utilization of 4 and 6 carbon fatty acids for growth and respiration in the phytoflagellate, *Polytoma uvella* strain 1,[†] it was shown that adaptation occurred in non-proliferating cell suspensions(1). The adaptation, therefore, was not dependent upon the development or selection of mutants. In non-proliferating cell suspensions an exogenous nitrogen source (NH_4Cl) was required, and the rate and final level of adaptation was quantitatively dependent upon the concentration of exogenous nitrogen (2). The fact that non-proliferating cells can adapt to fatty acid (oxidative) utilization, coupled with the absolute nitrogen requirement, indicated that the adaptation resulted from the induction of adaptive enzyme synthesis. This observation would extend the occurrence of this adaptive mechanism to another group of microorganisms in which it had not yet been demonstrated(3).

Ultraviolet irradiation, at appropriate intensities, has been shown to inhibit selectively the induction of enzymatic biosynthesis without affecting the activity of pre-existing enzymes(4,5). The present report describes the selective inhibition by ultraviolet irradiation of the induced synthesis of the butyric acid oxidase system in non-proliferating suspensions of *P. uvella,* strain 1.

Materials and methods. To study the adaptation to butyric acid oxidation, the cells were grown at 30°C on an acetate medium (modified from(6)) of the following composition: a. Buffered salt base: Ethylene diamine tetraacetic acid (0.01 g), K_2HPO_4 (0.004 g), $MgSO_4 \cdot 7H_2O$ (0.016 g), $ZnSO_4 \cdot 7H_2O$ (0.022 mg), $CaCl_2$ (0.006 mg), H_3BO_3 (0.012 mg), $MnSO_4 \cdot 4H_2O$ (0.005 mg), $FeSO_4 \cdot 7H_2O$ (0.004 mg), $Na_2MoO_4 \cdot 2H_2O$ (0.0015 mg), $CuSO_4 \cdot 5H_2O$ (0.0013 mg), $CoSO_4 \cdot 7H_2O$ (0.002 mg), Tris (hydroxymethyl) aminomethane (0.121 g); distilled water 100 ml; pH 8. b. Substrate: NaAcetate-$3H_2O$ (0.02 M) and c. Nitrogen source: NH_4Cl (50 mg). A 24 hour culture of acetate grown cells (in the logarithmic phase) was concentrated, washed by centrifugation and suspended in the buffered salt base of the growth medium. The suspensions were placed in standard, two-side-arm, 15 ml Warburg vessels (final volume 3.0 ml). Oxygen consumption was measured at 30°C with 20% KOH in the center well. Substrates and nitrogen source were placed in the side-arms, and added to the main chamber at zero time (after a 10 minute equilibration period). In irradiation studies, the cells to be irradiated were placed in petri dishes, suspended in the buffered salt base, and exposed to irradiation from an Ultra-Violet Products Inc., Mineral

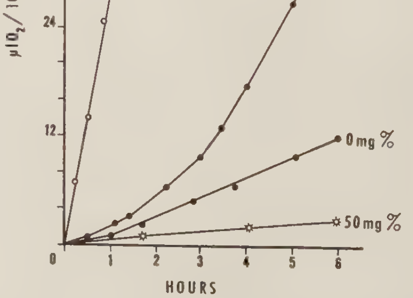

FIG. 1. Effect of exogenous nitrogen on butyrate adaptation. Concentration of NH_4Cl is shown for each curve.

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[*] This investigation supported, in part, from Research Committee of University of Oklahoma School of Medicine.

[†] *Polytoma* is a colorless (non-photosynthetic) flagellate classified both as member of Order Volvocales in Chlorophyceae and Order Phytomonadida of Class Phytomastigida by phycologists and protozoologists, respectively.

Light set at a 20 cm distance above the cells (principal wavelength at 2540 mu). After irradiation, the irradiated cells and the non-irradiated controls were added to Warburg flasks, and oxygen consumption was measured as before, except that irradiated cells were handled in dim light to minimize the possibility of photoreactivation.

Results. Adaptation to butyric acid oxidation by acetate grown cells does not occur in the absence of exogenous nitrogen, so that the pre-adaptive level of oxidation does not increase above the initial rate (Fig. 1). A concentration of 50 mg % NH_4Cl permits a 600% increase in the rate of butyric acid oxidation in 6 hours (Fig. 1). That this occurs without cellular proliferation was shown by direct cell counts before and after each experiment. However, in the presence of an exogenous nitrogen source, an increase in cell mass would be expected to occur even in the absence of cell division. The extent of cellular increase could be measured by the increase in activity of constitutive enzymes. Since *Polytoma* is a member of the "acetate flagellates" which utilize acetate preferentially and constitutively(6), acetate oxidase activity should be a faithful index of the increase in cell mass in the absence of cellular proliferation. During intervals of observation of from 6 to 8 hours, the rate of acetate oxidation does not increase by more than 25% while the increase in butyric acid oxidation increases by 600%.

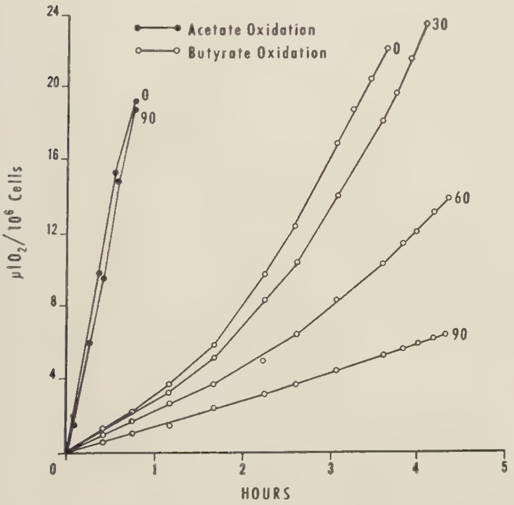

FIG. 2. Effect of ultraviolet irradiation on oxidative of acetate and adaptation to butyrate. Irradiation time, in sec, is shown for each curve.

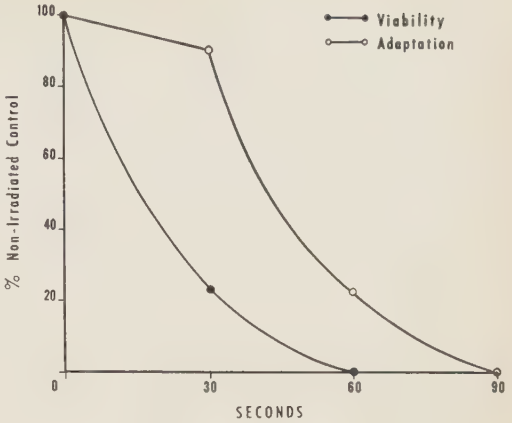

FIG. 3. Differential inhibition by ultraviolet irradiation of butyrate adaptation and viability. Abscissa represents time of irradiation (in sec) and ordinate the rate of butyrate oxidation as compared with non-irradiated control.

Ultraviolet irradiation at 30, 60, and 90 seconds progressively inhibits butyrate adaptation, the resulting inhibitions (measured as the oxidative rate on the adaptive substrate at the end of 4 hours) being 10% at 30 seconds, 67% at 60 seconds, and 99% at 90 seconds. These levels of irradiation do not affect the rate of acetate oxidation (Fig. 2).

A comparison of the relative effect of irradiation on viability versus adaptation of the cells used in the previous experiment is shown in Fig. 3. The ability to dissociate viability from adaptation at low intensities of ultraviolet is in confirmation of previous studies with other microorganisms(5,7).

Discussion. These data are presented to demonstrate the occurrence of induced adaptive enzyme synthesis in the fatty acid metabolism of a phytoflagellate. The occurrence of this adaptive mechanism is, therefore, extended to still another taxonomic group of microorganisms.

The ultraviolet data are pertinent in respect to the irradiation sensitivity of different levels of cellular activity. Ultraviolet irradiation effectively separates viability, adaptability (adaptive enzyme synthesis), and the enzymatic activity of constitutive enzymes which represent 3 progressively higher degrees of ultraviolet sensitivity. Ultraviolet intensities which markedly reduce viability affect adaptive enzyme synthesis only slightly, and have no effect on the oxidative activity of constitutive enzymes (Fig. 2). Higher intensities (30 to 90 second exposures) progressively reduce adaptive enzyme synthesis until it is inhibited completely without affecting constitutive enzyme activity. This pattern of progressive sensitivity of viability, adaptability and oxidative activity by constitutive enzymes, in that order, already has been reported for yeasts(7) and bacteria(5). The extent to which ultraviolet irradiation inhibits adaptive enzyme synthesis by interfering with nitrogen assimilation is still under study. However, preliminary, unpublished data indicate that ultraviolet does not interfere with nitrogen assimilation at intensities which inhibit the induction of adaptive enzyme synthesis.

Summary. Induction of adaptive enzyme synthesis is described in a phytoflagellate. The adaptation has been shown to occur in non-proliferating cell suspensions, and it is also shown to be dependent upon an exogenous source of nitrogen. The adaptive enzyme synthesis is selectively inhibited by ultraviolet irradiation without affecting the activity of pre-existing enzymes. At low ultraviolet intensities viability and adaptability have been partially dissociated; adaptation is only slightly inhibited while viability is reduced by 80%.

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Received January 31, 1955. P.S.E.B.M., 1955, v88.

Effect of Potassium Iodide on Plasma Cholesterol of Rats. (21587)

RICHARD S. HOMER,* (Introduced by Meyer Friedman.)
From Harold Brunn Institute, Mount Zion Hospital, San Francisco, Calif.

Liebig(1) observed that administration of iodides (organic or inorganic) to cholesterol fed rabbits appeared to protect them against occurrence of atherosclerosis. This observation has been confirmed by various observers (2), although there has been some disagreement(3). Whatever this mode of protection against atherosclerosis might be, it seems that relative protection of such rabbits against hypercholesteremia is one of the preventive factors(2,4). It is only fair to add that some workers have not found this hypercholesteremia protecting effect of iodides in rabbits (3,5). The possible critical importance of dosage(6) may be the reason for these discrepancies. Moreover, this antihypercholesteremic effect of KI could not be confirmed when chickens were used(7).

Since there appear to be fundamental differences in cholesterol metabolism between rats and rabbits(8), it was decided to study the effect of KI on plasma cholesterol of rats under various conditions. As a preliminary step, we determined the effect of KI on plasma cholesterol of rats deprived of external sources of cholesterol.

Methods. Male Long-Evans rats approximately 12 weeks old were divided into 3 groups and placed on fat and sterol-free diet for the duration of the experiment. After 24 hours on this diet, all rats were bled from the tail for initial plasma cholesterol determina-

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* Public Health Service Research Fellow, of National Heart Institute, National Institutes of Health. Aided by a grant from Life Insurance Medical Research Fund.

TABLE I. Effect of Tube Feeding KI, H_2O and KCl upon Plasma Cholesterol of Rats on Sterol Free Diet.

Material fed	No. of rats	Avg wt, g	Avg plasma cholesterol: First day pre-tube feeding, mg/100 cc	Avg plasma cholesterol: Third day, mg/100 cc
KI*	25	230 (180-260)§	48 (33-67) ± 2.18‖	92 (67-130) ± 3.45
H_2O†	23	232 (198-262)	44 (20-65) ± 2.43	60 (42-89) ± 2.53
KCl‡	10	220 (192-232)	51 (37-59) ± 2.93	64 (48-74) ± 2.81

* KI = 200 mg/day (1 cc), 3 days.
† H_2O = 1 cc/day, 3 days.
‡ KCl = 200 mg/day (1 cc), 3 days.
§ Range of values.
‖ Stand. error of mean.

tion. Beginning that day and for 3 days, the rats were anesthetized and fed test materials by stomach tube. The experimental rats were fed 200 mg KI (200 mg/cc) daily; control rats received 1 cc H_2O daily; and the third group received 200 mg KCl (200 mg/cc) daily. Three hours after the last tube feeding (third day), the rats were again bled for plasma cholesterol determination.

Results. The change in plasma cholesterol concentration following tube feeding of the 3 test materials is shown in Table I. Although plasma cholesterol concentration rose significantly in all 3 series, after feeding period of 3 days, plasma cholesterol rise in the series given KI was considerably greater than in the other 2 series. The series given H_2O exhibited about as great a rise (16 mg/100 cc) as that observed in the series given KCl (13 mg/100 cc). The rise of plasma cholesterol observed in rats given KI was 44 mg/100 cc. The difference in the rise of plasma cholesterol between rats given KI and either of the other two groups is statistically significant well beyond the 1% level.

Discussion. It appears safe to conclude that the rise in plasma cholesterol following feeding of KI to rats, as described, is of such magnitude that iodide must be considered as an important hypercholesteremic agent. The slight rise in plasma cholesterol after administration of either KCl or H_2O alone is most likely due to non-specific factors such as repeated anesthesia, intubation, and dietary change rather than to substances fed. Turner also was unable to show any effect in rabbits with halides of potassium other than iodide.

This seemingly paradoxical hypercholesteremic effect of KI is perhaps not too unexpected in view of the fact that Turner and Bidwell (9) and Meeker, Kesten and Jobling(10) have demonstrated that administration of KI could maintain a hypercholesteremic state in hypercholesteremic rabbits, even after removal of dietary cholesterol. Although the actual mechanism by which iodides exert their effect is still unknown, many workers (2,3,6) have held that iodides produce their effect by altering thyroid function. On the other hand, other studies have shown that the effect of KI is unchanged in thyroidectomized rabbits(4,11). If the hypercholesteremic effect of KI in the rat were due to its action on thyroid, then appropriate changes in hepatic synthesis, destruction and intestinal excretion of cholesterol, as found in deranged thyroid states(12), should also be found in KI fed rats. Such possible changes, together with possible alteration of the physical state of plasma cholesterol as observed after administration of Triton WR-1339(13) and cholic acid(14), are now under investigation.

Summary. Evidence is presented which indicates that KI (200 mg/day for 3 days), when administered to rats on a sterol free diet, produces an hypercholesteremia of moderate degree.

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Received January 31, 1955. P.S.E.B.M., 1955, v88.

Transintestinal Intubation: Technic for Measurement of Gut Length and Physiologic Sampling at Known Loci.* (21588)

DAVID H. BLANKENHORN, JULES HIRSCH, AND EDWARD H. AHRENS, JR.
(Introduced by Vincent P. Dole.)
From the Hospital of The Rockefeller Institute for Medical Research, New York City

In a study of fat digestion in human subjects(1) it was considered desirable to sample intestinal contents in a precise but more physiological manner than was feasible with existing technics. Current widespread use of plastic tubes for gavage feeding of premature infants and of comatose patients suggested that such tubing might be suitable for aspiration as well. Polyvinyl tubing, which is more flexible than polyethylene, proved ideal for our purpose. The present report describes an intubation technic which has permitted numerous aspirations at various known levels in the gastrointestinal tract of a given subject. The procedure is patterned upon the technic of van der Reis and Schembra(2), who passed rubber tubes through the entire intestinal tract of animals and human subjects for purposes of measuring gut length. In the present method intubation is accomplished with negligible discomfort to the patient and with no evidence of alteration in gastrointestinal morphology or function.

Construction of Tube. The plastic tubing† is conveniently used in lengths of 10 m. One end of such a tube is doubled upon itself for 2 cm and tied with 2 sutures (surgical silk, size 000). A rubber bag‡ secured proximal to this doubled end (Fig. 1a) will not slip off the tube. 1-2 ml of reagent grade mercury is injected into the balloon through a #25 needle. To prevent trapping of gas in the balloon, several punctures of the balloon are made with a #25 needle, and the balloon tied on the tube over a #21 needle which is then withdrawn, as described by Cantor(3). All

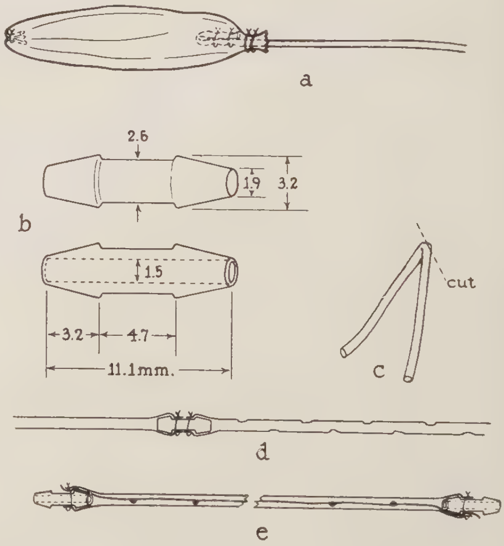

FIG. 1. Technical details of construction of transintestinal tube. a) method of securing balloon to tube; b) steel adapters, with and without lumen; c) method of cutting holes in tube for aspiration; d) method of connecting tubes over adapters; e) marking segment containing knotted radio-opaque suture material.

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* The authors acknowledge exceedingly helpful roentgenologic advice of Drs. Ross Golden, Edith Quimby, John Evans, and John McClenahan.

† Polyvinyl tubing, 2.1 mm OD, 1.5 mm ID, accepts No. 14 needle. Pharmaseal Co., Glendale, Cal., No. EHR-11, available in 500 foot lengths or less.

‡ Finger cot, or balloon for Miller-Abbott tube (No. P-9140, George P. Pilling and Son, Philadelphia, Pa.) with distal hole closed by suture and balloon inverted.

air remaining in the balloon is then expelled by manual pressure. Successive lengths of tubing are connected by steel adapters (Fig. 1b) over which tubing is secured with 2 silk sutures (Fig. 1d). Aspirating holes (2 mm) are cut in the tubing at any desired location by doubling the tube over and cutting off a small corner (Fig. 1c). The marking segment referred to below is made from 2 steel adapters, 2 m of tubing, and radio-opaque suture material[§] (Fig. 1e). A piece of this suture is sucked through the tubing by ordinary laboratory vacuum and is then used to pull into the tube a similar length of suture knotted at 15-20 cm intervals. The ends of the segment are closed by means of adapters such that the knotted suture lies within the tube and firmly fixed at each end between the adapter and the tube. The tubing is fixed to each adapter with silk sutures. *Passage of Tube.* The mercury-filled bag is passed through either nostril into the stomach by the Cantor technic (3). Local analgesia and icing of the tube have been unnecessary, but lubrication with jelly is helpful. The remaining tube is coiled and placed in the breast pocket of the patient's pajamas and *is not secured at the nose at any time.* The patient remains ambulatory and is given an ordinary hospital diet of solid foods. No special measures have been needed to hasten the passage of the tube through the pylorus; this occurs shortly after the next meal or sooner. The mercury-filled bag is allowed to pass through the entire intestinal tract (2-6 days) and upon delivery at the anus is cut off leaving a short length of tube which is brought up between the buttocks and taped over the sacrum. It is common for a loop of tubing to be passed per rectum before the balloon itself; by gentle traction the extra tubing and balloon are readily delivered. Tubing passed with each bowel movement is cut off, and the tube to be taped to the patient is cleaned with soap and water or mineral oil. Patients are encouraged to shower or bathe each day; no special hygienic problems have arisen either in male or female subjects.

Equilibration Period. Within 2 or 3 days after the passage of the balloon the length of tubing from nose to anus becomes fairly constant. In some patients transintestinal passage of the mercury-filled balloon is accompanied by considerable initial shortening of the gut upon the tube; after delivery of the balloon this shortening is spontaneously corrected by ingress of tube at the nose without comparable egress at the anus. In other patients, particularly those who are constipated, several loops of tube may pass into the large bowel before the balloon end is delivered through the anus; thereafter, more tubing is passed per rectum than is taken in at the nose. The end of the equilibration period occurs when the internal length of tube remains constant within ± 5% on 2-3 successive days. At that time withdrawal of 1 m of tube per rectum causes a comparable length to pass into the nose. This observation can be repeated several times in a day in order to establish completion of equilibration. Occasional patients complain of rectal discomfort during this maneuver, due to the pressure of fecal boli adherent to the tube; it can be prevented by a preliminary enema. Tubing passed or withdrawn per rectum has never been observed to re-enter the alimentary canal. After equilibration the tube migrates through the intestine at a rate of 30-100 cm per day. Our subjects have been placed on complete liquid formula feeding by mouth as soon as the balloon has been passed per rectum. This dietary change serves 3 purposes: 1) patients can be acclimated for several days to a dietary intake similar or identical to the test meal; 2) absence of grossly particulate foods permits representative samples of chyme to be aspirated rapidly despite the small size of the perforations and lumen of the tube; and 3) complete feeding can be accomplished with a small number of well-defined food elements, thus greatly simplifying the chemical analysis of withdrawn samples. Many considerations have, in fact, led us to the use of orally-fed liquid formulas for complete maintenance of patients under metabolic study; more than 50 patients have been so maintained for periods of 1-12 months with no detectable impairment of

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[§] Same as sutures in Ethicon Ray-tec x-ray detectable sponges, Johnson and Johnson, Inc., New Brunswick, N. J.

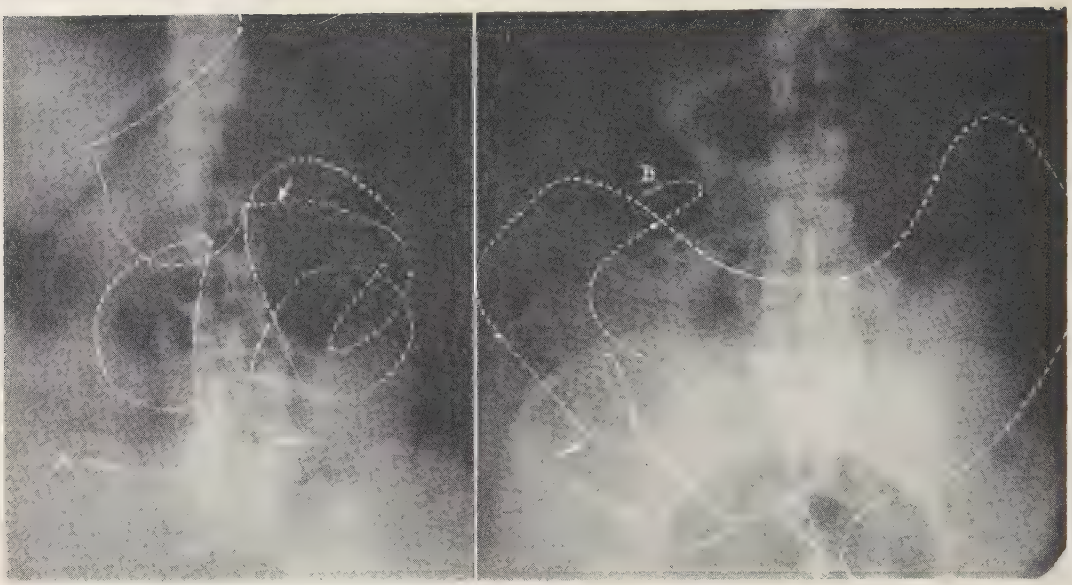

FIG. 2. Location of anatomical landmarks by means of marking segment. a) Flat plate taken when adapter B reached nose showed marking segment in stomach and upper intestine with adapter A in jejunum. Arrow points at location of ligament of Treitz where jejunum becomes freely movable. b) Flat plate taken when adapter A appeared at anus showed marking segment in terminal ileum and colon, with adapter B in ileum. Arrow points at angulation of tube at ileo-cecal valve.

health. Our technic of oral formula feeding has been described elsewhere(4).

Location of Anatomical Landmarks. In order to place aspirating holes at known loci in the intestine, a method was developed for measuring in each patient the distances from nose to pylorus, to duodeno-jejunal junction (ligament of Treitz), to ileo-cecal valve and to anus. After the tube is passed per rectum and the period of equilibration completed, the tube is cut near the nose, and the marking segment described above is spliced into the gap. The first adapter of the marking segment to pass into the nose is called adapter A and the second, adapter B. A record is made of 1) the length of the marking segment, 2) the length of the tube fastened to adapter B, and 3) the distances between knots in the radio-opaque suture within the marking segment. The tube is then permitted to pass at its own rate into the gut. Two x-ray flat plates of the abdomen are taken, the first when adapter B is at the nose and the second when adapter A appears at the anus. The 2 films will show the segment stretching from nose to upper jejunum and from lower ileum to anus, respectively. Fig. 2 illustrates 2 such flat plates in the same patient; the radio-opaque suture and its knots are clearly seen in the original x-rays. In Fig. 2a the distance from nose to adapter A was found to be 183 cm by subtracting the length of tube outside

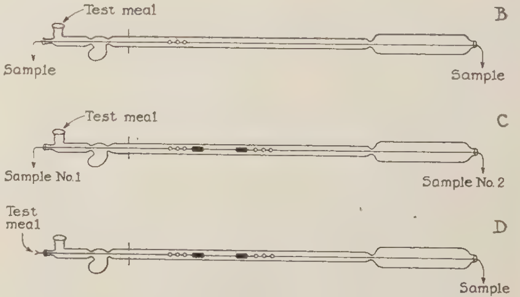

FIG. 3. Schematic representation of various ways in which transintestinal tube can be used for sampling intestinal contents.

TABLE I. Clinical Data on 12 Adult Patients Subjected to Transintestinal Intubation. None had clinical or roentgenologic evidence of gastro-intestinal dysfunction.

Patient	Age	Sex	Clinical diagnosis	Clinical status	Initial transit time, nose-to-anus (days)	Total time intubated (days)
1	29	♂	Obesity	Am†	3	10
					6	26
2	43	♂	Arteriosclerotic heart disease	Am, no an	2	9
3	37	♀	Chronic anxiety state	Am	6	18
4	54	♂	Arteriosclerotic heart disease	Am, moderately severe an	6	21
5	53	♂	" " "	Am, no an	2	18
6	63	♂	" " "	Am, no an	5	18
7	37	♀	Obesity	Am	2	21
8	22	♀	"	"	2	14
					3	17
9*	74	♂	Cerebral vascular accident, old	Hemiplegic, wc	3	10
10*	78	♂	Chronic pyelonephritis	wc	4	10
11*	42	♂	Subarachnoid hemorrhage, recent	Bedfast	3	4
12*	21	♀	Infectious hepatitis	"	4	8

* Patients 9-12 intubated by resident staff at Cincinnati General Hospital under supervision of first author. Other patients intubated at Rockefeller Institute Hospital.

† Am = ambulant; an = angina; wc = wheel chair.

the nose from the total length of tube attached to adapter A of the marking segment. The distance from adapter A to the ligament of Treitz was estimated to be 107 cm since this point was seen to be equidistant between knots known to be 97 and 117 cm from adapter A. Therefore, the distance from nose to ligament of Treitz was 183 – 107 or 76 cm. The distances from nose to pylorus (58 cm), to ileocecal valve (394 cm), and to anus (500 cm) were derived similarly. From these data the lengths of the duodenum, jejuno-ileum, and large bowel were calculated. *Sampling.* When the anatomical landmarks have been identified as described, the tube is cut near the nose, a closed adapter is secured between the cut ends, and holes are cut at 0.5-1.0 cm intervals in the 10 cm segment following the closed adapter (Fig. 1d). The tube is allowed to migrate through the nose the calculated number of centimeters until the holes have reached the location in the gut where sampling is to be carried out. During sampling periods of 2-4 hours the tube is usually stationary but may migrate as much as 30 cm. We have preferred to permit this movement rather than to incur the greater disadvantage of unphysiologic shortening of bowel upon a fixed tube. Samples of contents from the upper intestine have been obtained by simple siphonage at a rate of 1-200 ml per hour; 10 ml of water is injected into the tube hanging free at the nose, and the water-filled tube is hung over the side of the bed or chair to the receiving flask at floor level. Occasionally it is more efficient to use a suction pump‖ at 70 or 100 mm of mercury pressure. Pharmaseal tubing does not collapse under this degree of negative pressure, nor under the pressures produced during vigorous syringe aspiration. The collection of chyme in polyethylene bottles¶ immersed in dry ice and acetone in a wide-mouthed thermos insures inhibition of enzymatic activity. Samples can be stored frozen until studied further. Several alternative sampling procedures have been used (Fig. 3). (A) With test formulas fed by mouth, the intestinal contents have been sampled at any desired point in the intestine. Then, after permitting free migration of the tube, test feedings and aspirations have been repeated at lower intestinal levels. In one subject 6 samples were obtained over a 4-day period at various points from duode-

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‖ Gomco Surgical Manufacturing Corp., Buffalo, N. Y., thermotic drainage pump No. 763.

¶ Arthur H. Thomas Co., Philadelphia, Pa., No. 2205-A.

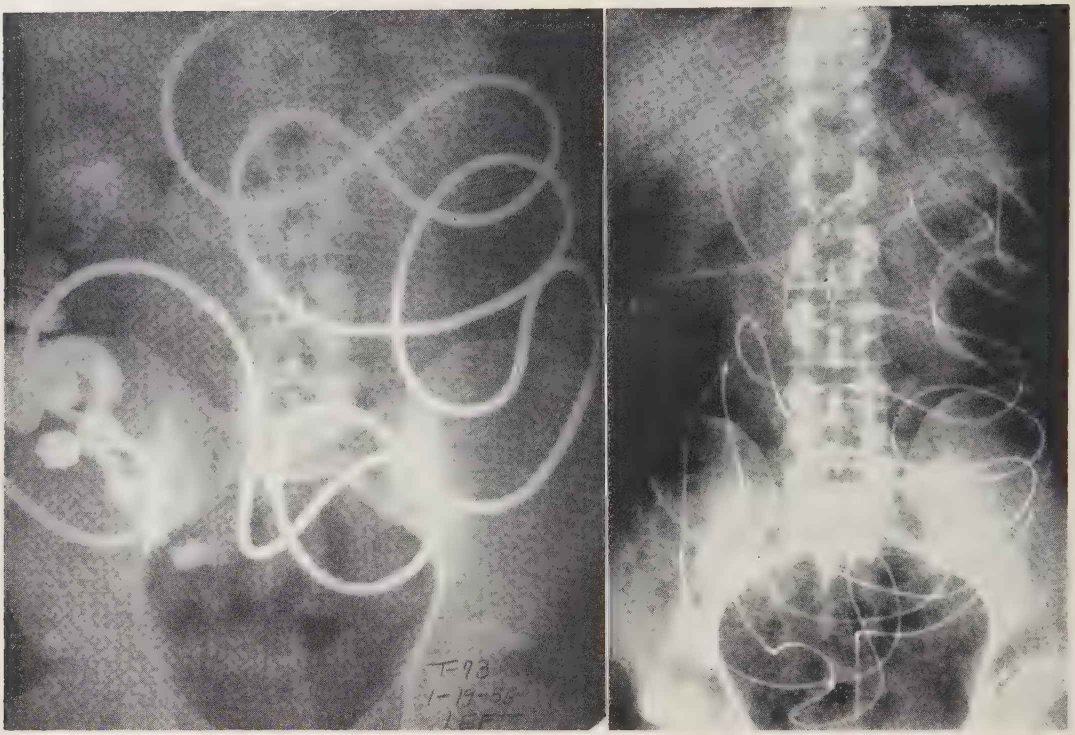

FIG. 4. Configuration of intestinal loops intubated with Miller-Abbott tube and polyvinyl tube in 2 patients with normal intestinal tracts. Left—Miller-Abbott tube remained at ileo-cecal valve for 4 days without passing into colon; barium injected through tube fills the colon. Right—Polyvinyl tube through entire intestinal tract is filled with barium.

num to lower ileum. (B) The yield of sample can be increased by aspirating intestinal contents simultaneously from both ends of the tube, since the rapidity of drainage is influenced more by the frictional resistance of the tube than by the size and number of aspiration holes. (C) By incorporating a blind segment in the tube between 2 closed adapters, with holes above and below this segment, aspirations have been made simultaneously at 2 points of known distance from each other. (D) With the same arrangement of tubing the test formula has been introduced through the upper holes while sampling was carried out from the lower holes. Conversely, reflux movement of intestinal contents can be studied by injecting formula through the lower holes while aspirating from the upper holes. It is possible that the technic of landmark-location combined with local injection of contrast medium would be useful in the roentgenologic localization of pathologic conditions of the intestine.

Withdrawal of Tube. After completion of tests any tube remaining outside the nose is cut off close to the nostril. The transintestinal tube is allowed to migrate caudally until all of it has been passed per rectum. Passage of the tube can be hurried by repeated gentle traction on the anal end and by ingestion of solid foods. *Limits of X-ray Irradiation.* On the advice of radiologists we have limited the taking of abdominal flat plates in any given adult patient to 15 films over a 2-week period, with 35 by 43 cm (14″ x 17″) plates exposed for 0.3-0.5 sec. at 65-90 kv., using a 1.0 mm aluminum filter. With our apparatus the patient receives less than 2 r per film or 30 r per 2-week period. We have not found fluoroscopy to be necessary or helpful in these studies. *Precautions.* Patients are advised to light cigarettes cautiously, because the polyvinyl tubing can be made to burn in a direct flame. Tubes *must* be tied securely over the adapters with surgical silk, square-knotted; such joints are as strong as the tube itself.

Kinking and knotting of the tube in the large bowel has occurred rarely but does not pose a problem because of the pliability of the tube. We have not attempted transintestinal intubation in patients with esophageal varices or with known obstructive, ulcerative, or inflammatory lesions of the alimentary canal.

Results. Twelve adult patients have been intubated for periods varying from 4-26 days with a total of 204 man-days of intubation. Relevant clinical data are listed in Table I. There was no clinical or roentgenologic evidence of gastrointestinal dysfunction in any of the subjects. The procedure was well tolerated in all cases. All complained of slight irritation in the intubated nostril and in the posterior pharynx for 2-3 days; this spontaneously disappeared in all patients. Local medication with neosynephrine nose drops and with nupercaine lozenges was helpful during this period. Throat discomfort attributable to passage of adapters was prevented by spraying the throat with 1% pontocaine as the adapter approached the pharynx. On 2 occasions nasal congestion necessitated changing the tube from one nostril to the other. Patients became acclimated to the procedure very quickly, carried on their previous activities, slept well, and maintained weight on their previous food intakes. Bowel movements occurred without change; stools were guaiac-negative. Nausea and vomiting, so frequently encountered during experimental procedures with other tubes, were completely absent. Although this clinical experience was devoted primarily to perfecting details of the intubation procedure, 25 aspirations of intestinal contents have been carried out satisfactorily in 7 patients. Definition of the hydrolytic products of triglyceride digestion in Patients 1 and 2 have been published elsewhere (1).

The configuration of the tube within the abdomen was demonstrated radiographically by injecting a thin suspension of barium through it or by using a tube fully lined by radio-opaque suture material. Fig. 4 contrasts the large number of small loops taken by the plastic tubing with the relatively uncoiled inflexible appearance of a Miller-Abbott tube. When barium was swallowed in

TABLE II. Intubation Data on 8 Patients with Calculated Lengths of Various Gut Segments.

Patient	Body ht (cm)	Body wt (kg)	Tube length, cm				Calculated lengths of segments, cm			Ratio of nose-anus length to body ht	Ratio of jejuno-ileum/nose-to-anus lengths
			Nose to anus	Nose to pylorus	Nose to ligament of Treitz	Nose to ileo-cecal valve	Duodenum	Jejuno-ileum	Colon		
1	175	127	478	*	86	371	*	285	107	2.75	.595
2	168	68	434	*	91	331	*	240	103	2.58	.541
3	155.5	70.5	394	51	74	279	23	206	114	2.54	.522
4	160	53	412	*	81	297	*	216	114	2.57	.522
5	157.5	58.8	441	74	100	350	26	250	91	2.80	.569
6	176	78.5	536	74	94	411	20	317	125	3.05	.592
7	158	101.7	500	58	76	394	18	318	106	3.16	.635
8	154	98.8	417	*	64	295	*	231	122	3.69	.552

* Not measured.

order to delineate mucosal patterns around the plastic tube, normal radiologic appearance of the small bowel was found in the 5 patients so examined.

Lengths of various portions of the gut measured in 8 of the patients are listed in Table II. With this technic the ratio of nose-anus length to body height was about 2.5 to 3.5, while the proportion of the gut represented by the jejuno-ileum was slightly more than 50% of the total gut length. Day-to-day reproducibility of total gut length can be illustrated by measurements made in Patients 1 and 8, in whom there was no manipulation of the tube for several successive days. In Patient 1 the nose-anus lengths were 465, 492, 461, 442, 467, 442 and 477 cm; in Patient 8, 424, 391, 414, 401, and 409 cm. Thus, the total gut length as measured in this manner varied by ± 5% or less. The relationship between the measurements shown in Table II and the true lengths of unintubated gut segments will be discussed in greater detail in a later report(5).

Summary. A technic is described for sampling the contents of the intestinal tract in human subjects repeatedly and at various intestinal levels over a 1-2 week intubation period. Prolonged transintestinal intubation with polyvinyl tubing (2.1 mm OD) has been well tolerated, and sampling has been effected under conditions which closely approach the physiological state. A means of localizing the site of aspiration is described. Reproducible measurements of the length of the intestinal tract have been made in 8 adult patients.

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Received January 31, 1955. P.S.E.B.M., 1955, v88.

Induction of Parotid Carcinomas and/or Subcutaneous Sarcomas in C3H Mice with Normal C3H Organ Extracts.* (21589)

LUDWIK GROSS.

From the Cancer Research Unit, Veterans Administration Hospital, Bronx, New York City.

Leukemia or parotid tumors (in a few instances subcutaneous sarcomas) could be induced following inoculation of centrifuged or filtered Ak leukemic or Ak normal tissue extracts into newborn mice of the C3H strain (1-5). This report deals with experiments devised to recover the causative agents from induced leukemic or parotid tumors. Furthermore, extracts from C3H embryos, normal organs, also sterile broth and physiological saline solution, were inoculated into newborn C3H mice.

Materials and methods. Extracts of 20% concentration prepared with physiological saline solution(3-5) from leukemic organs, parotid tumors, or normal organs, were centrifuged at 3000 RPM (1400 x g) for 15 minutes, then at 9500 RPM (7000 x g) for 10 minutes, at 0°C. The supernate was used for inoculation, or passed through a Berkefeld-N, or Selas 03 filter, prior to use. All extracts were kept at 0°C and injected (0.1 cc, subcutaneously) within 48 hours. *Test animals.* Newborn C3H, or foster nursed C3H(f) mice, both of the Bittner substrain(6) were used for inoculations. Most animals were less than 12 hours, many less than 6 hours, none more than 15 hours old, at time of inoculation.

Results. Pathogenic properties of extracts prepared from induced C3H leukemias. For these extracts we used mediastinal and mesenteric tumors, spleens and livers of leukemic

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* Aided, in part, by grant from Damon Runyon Memorial Fund for Cancer Research.

TABLE I. Results of Inoculation of Centrifuged or Filtered Extracts from Induced C3H Leukemia into Newborn (<15 Hr Old) C3H or C3H(f) Mice.

No. of exps.	Donor's leuk. resulted from inoc. of:	Strain	No. of mice inoc.	No. of mice developing*		
				Leuk.	Parot. tumors	Sarc.
18	Ak leuk. filtr. or centr.		69	19	5	1
8	Transpl. (8 to 15×) C3H leuk. cells		21	0	7	2
3	Parot. tumors filtr.		7	2	0	0
2	C58 leuk. filtr.		8	4	0	0
31		C3H	66	13	9	3
		C3H(f)	39	12	3	0
		Total	105	25†	12‡	3

* Avg age leukemia developed 5 mo, parot. tumors 4 mo, sarcomas 8 mo.
† 2 of these mice developed leukemia and also bilateral parotid tumors.
‡ 4 mice developed parotid tumors and also subcutaneous sarcomas.

C3H mice. In most animals leukemia resulted from the previous inoculation of cell-free (centrifuged or filtered) Ak leukemic extracts. In some experiments, initially induced leukemia in C3H mice was transplanted in mice of the same line, and cell-free extracts were then prepared from intermediate and 15th consecutive transfers. In a few experiments, we used as donors C3H mice, in which leukemia had been induced with filtered C58 leukemic extracts, or with extracts prepared from C3H parotid tumors. Of the 105 newborn C3H, or C3H(f) mice inoculated with centrifuged or filtered C3H leukemic extracts, 25 developed leukemia, 12 parotid tumors, and 3 subcutaneous fibrosarcomas; among 12 mice with parotid tumors, 4 developed in addition subcutaneous sarcomas (Table I).

Pathogenic properties of extracts prepared from induced parotid tumors. In this series the donors were C3H mice with induced or transplanted parotid tumors. The induced parotid tumors resulted from inoculation of filtered, or centrifuged Ak or C58 leukemic extracts, or of extracts prepared from normal Ak organs. Some of these parotid tumors were transplanted subcutaneously in C3H mice. Centrifuged or filtered extracts were then prepared from several intermediate and 18th consecutive transfers. These parotid tumors were removed aseptically, ground with sterile physiological saline solution to obtain 20% concentration, and centrifuged. Of 296 newborn C3H or C3H(f) mice, inoculated with either centrifuged or filtered parotid tumor extracts, 24 developed leukemia and 21 parotid tumors; 6 of these latter mice developed also subcutaneous fibrosarcomas; 2 mice developed tumors of the parotid and submaxillary glands, and also in the adrenal medulla (Fig. 1). Nine mice developed only soft tissue fibrosarcomas with no evidence of either leukemia or parotid tumors. (Fig. 2 and 3)

Results of inoculation of extracts prepared from normal C3H and C57 Brown/cd organs. To determine whether extracts from organs of normal, healthy mice of a low-leukemic line may also induce leukemia or tumors, centrifuged extracts were prepared from *normal embryos* removed aseptically from young,

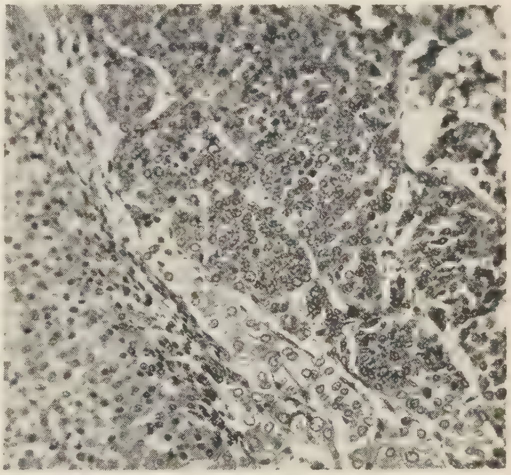

FIG. 1. Medullary adrenal tumor. This C3H female was inoculated when less than 4 hr old with centrifuged extract from 18th transfer of a C3H parotid tumor. At 2½ mo of age this mouse developed bilateral parotid and also submaxillary gland carcinomas. A medullary adrenal tumor was also found. (H. & E. × 120.)

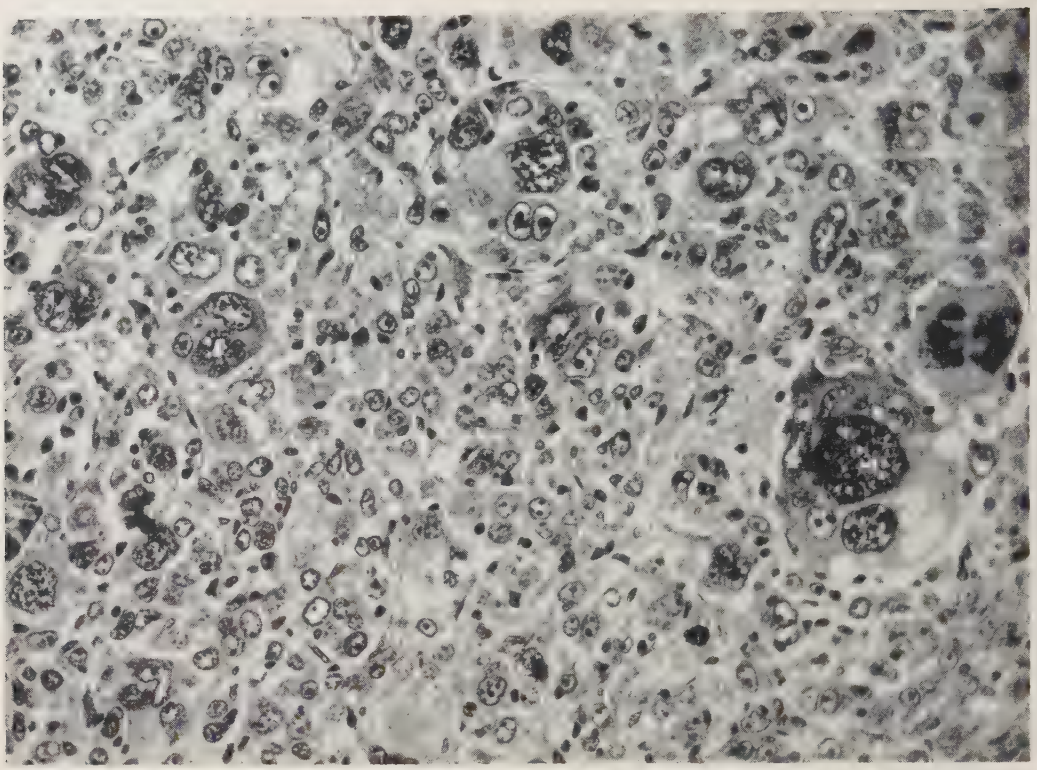

FIG. 2. Spindle and giant-cell sarcoma. This C3H male was inoculated when less than 9 hr old with centrifuged (7,000 x g) parotid tumor extract, and developed at 8½ mo a rapidly growing tumor infiltrating right front leg in the vicinity of axillary pit. This tumor could be readily transplanted to other C3H mice, retaining its peculiar morphology. (H. & E. × 330.)

normal pregnant female mice of the C3H line (Bittner substrain) mated to their brothers. In 2 experiments, extracts were prepared from normal C57 Brown/cd(5) embryos. Fifty-three newborn C3H and 50 C3H(f) mice (total 103) were inoculated with C3H embryo extracts, and 3 C3H mice developed parotid tumors at 4 to 12 months; no leukemia developed in any injected animal. Of 9 C3H mice inoculated with C57 Brown/cd embryo extracts, 1 developed a parotid tumor at 4½ months. In a second series, centrifuged extracts of *normal mammary glands* prepared from lactating C3H females were inoculated into 12 newborn C3H and 48 C3H(f) mice and 9 (4 C3H and 5 C3H(f)) developed parotid gland tumors at 3 to 8 months of age. Among these 9 mice, 1 C3H(f) developed also a subcutaneous fibrosarcoma. Four C3H(f) mice developed subcutaneous fibrosarcomas at 11½ and 15 months, respectively, without any evidence of parotid tumors. No leukemia appeared in any of the inoculated mice, but among the females several developed mammary carcinomas. In a *third series,* centrifuged extracts were prepared from *pooled normal organs* (spleen, liver, kidneys, heart, lungs) of young, healthy C3H(f) or of C3H mice. These extracts were freshly prepared and used for inoculation within 48 hours; or the extracts were preserved by dry-freezing, or in 50% glycerine, for 2 weeks to 2 years, then resuspended, and used for inoculation. Of 74 newborn inoculated C3H or C3H(f) mice, 10 developed parotid gland tumors (Table III), none developed leukemia. Parotid gland tumors, which developed in C3H or C3H(f) mice, following inoculation of normal C3H or C3H(f) or C57 Brown/cd organ extracts, were identical with those(4) in which Ak leukemic extracts were used for inoculation.

Results of inoculation of sterile physiological saline, or tryptophane broth into newborn C3H mice. In control experiments, ster-

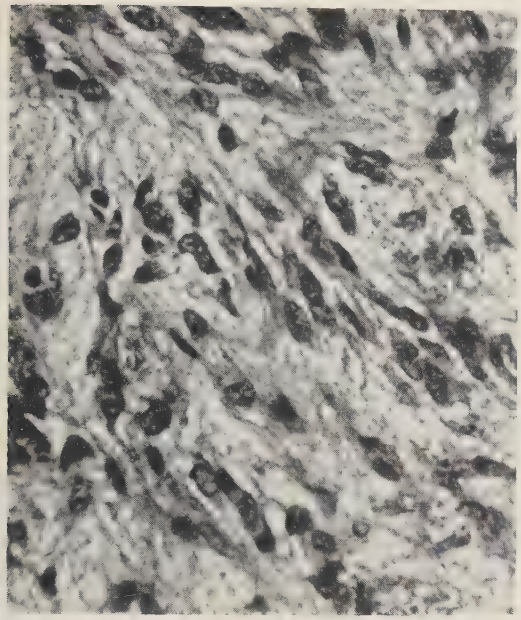

FIG. 3. Fibrosarcoma in a C3H male. This mouse inoculated when less than 3 hr old with filtered (Selas 03) parotid tumor extract. At 10 mo a hard tumor mass developed under the skin of back, growing progressively. (H. & E. × 342.)

ile physiological saline solution, or sterile tryptophane broth (cooled to 0°C) were inoculated subcutaneously into 42 newborn C3H and 20 C3H(f) mice. None developed either leukemia, parotid gland tumors, or subcutaneous sarcomas.

Discussion. Mouse leukemia in Ak and C58 inbred lines, is caused by a filterable and thermolabile agent, presumably a virus, transmitted through the embryos, and remaining latent until the carrier-mice reach middle age. Cell-free extracts from leukemic or healthy mice of either inbred strain, contain the agent: when inoculated into newborn mice of a susceptible "detector" strain, such as C3H (Bittner substrain), or C57 Brown/cd, the extracts may induce typical leukemia(1-7). Under certain conditions, inoculation of newborn C3H mice may result in development of parotid gland tumors, and/or subcutaneous sarcomas. Development of parotid tumors could be induced by inoculating (a) leukemic Ak extracts centrifuged at 7000 x g then diluted(7), (b) supernate resulting from centrifugation of Ak leukemic extracts at 144,000 x g(8), (c) leukemic extracts passed through filters such as Selas 03(4), or gradocol membranes(9), or (d) centrifuged organ extracts from healthy Ak or C58 carriers, such as extracts from Ak spleen, embryo, or breast tissue(9).

One may assume that a single agent present in Ak or C58 extracts (when inoculated into newborn C3H mice) may induce either leukemia or salivary gland tumors, or other tumors, such as soft tissue sarcomas. It is then difficult to explain, however, why such agent would very exceptionally induce in the same animals both leukemia and parotid tumors. We have seen only 3 such mice among several hundred inoculated. Also why would such agent never induce spontaneous salivary gland tumors in natural Ak or C58 carriers, and why would inoculation of C57 Brown/cd mice with Ak extracts result only in development of leukemia, but not parotid tumors (5)?

On the other hand, mice of high-leukemic Ak or C58 inbred lines may possibly carry not only a leukemic, but also other oncogenic agents, the latter not pathogenic for their natural Ak or C58 carriers. Such a working hypothesis appears consistent with the experimental facts. When Ak or C58 extracts, containing both agents, were inoculated into newborn C57 Brown/cd mice, only leukemia resulted. Mice of this strain may be susceptible to leukemic, but not to parotid tumor agent. When, however, these extracts were inoculated into newborn C3H mice, susceptible to both agents, either leukemia or parotid tumors (and in a few instances subcutaneous sarcomas) resulted. Among inoculated C3H mice some developed leukemia, and others parotid tumors. This could be due to an interference phenomenon. Only one of these two agents may usually induce the disease in one host, though both may be present. It follows, with few exceptions, that either leukemia or parotid tumors induced with Ak or C58 extracts in C3H mice, under conditions hitherto applied, would contain both agents. Such an assumption would be consistent with results reported in Tables I and II. Thus, when extracts prepared from induced C3H leukemias or from induced parotid tumors were inoculated into newborn C3H mice,

TABLE II. Results of Inoculation of Centrifuged or Filtered Parotid Tumor Extracts into Newborn (<15 Hr Old) C3H or C3H(f) Mice.

No. of exps.	Donor's tumors resulted from inoc. of:	Strain	No. of mice inoc.	No. of mice developing*		
				Leuk.	Parot. tumors	Sarc.
22	Ak leuk. filtr. or centr.		93	11	2	3
21	Ak leuk. centr. extr. dil. 1:1,000 to 1:100,000		76	2	2	3
10	Ak organs centr.		44	1	2	1
16	Induced parot. tum., centr., or transpl. (1 to 18×) parot. tum. cells		60	10	15†	1
3	C58 leuk. filtr.		14	0	0	1
1	C3H leuk. cells, transpl. (9×)		9	0	0	0
73		C3H	229	15	7	5
		C3H(f)	67	9	14	4
		Total	296‡	24	21†	9

* Avg age leukemia developed 13 mo, parot. tumors 5 mo, sarcomas 11 mo.

† 6 mice developed parotid tumors and also subcutaneous sarcomas; 2 mice developed parotid and submaxillary gland tumors, and also medullary adrenal neoplasms.

‡ In addition 35 newborn mice of the less susceptible C3H/An (National Cancer Inst.) substrain(6) were also inoculated: only one developed a uterine sarcoma, none parot. tumors or leukemia.

either leukemia or parotid tumors resulted. That extracts prepared from those parotid tumors, initially induced with highly diluted (1:1000 to 1:100,000) Ak leukemic extracts, rarely reproduced leukemia (Table II) may be significant. Previously, highly diluted Ak leukemic extracts induced parotid tumors, but rarely leukemia(7). It is conceivable that parotid tumors, induced with diluted leukemic extracts, contain mostly the parotid agent, and very little of the leukemic virus. For this reason cell-free extracts prepared from such parotid tumors may only very occasionally induce leukemia, whereas extracts prepared from parotid tumors induced with non-diluted leukemic extracts may reproduce either disease.

It was apparently much easier to recover either agent from induced C3H leukemias (Table I) than from parotid tumors (Table II). Whether leukemic tissues contain larger quantities of both agents than parotid tumors, or whether other reasons may be responsible for this difference, remains to be determined.

Untreated Ak or C58 mice have never developed parotid tumors spontaneously. When used as donors, however, for preparation of extracts, they represent an excellent source of the parotid tumor agent. It would appear, therefore, that mice of these 2 inbred lines are usually not susceptible to the pathogenic action of such an agent. As with certain phages(10), one could assume that the parotid tumor agent is "not inducible" in its natural Ak or C58 carrier. Ak and C58 mice are not necessarily the only ones carrying such a masked oncogenic agent. When extracts, prepared from normal organ extracts of healthy C3H, C3H(f) or C57 Brown/cd mice were inoculated into newborn C3H mice, parotid tumors were sometimes induced (Table III). This agent would therefore exist in C3H mice in a masked form, usually not pathogenic for its natural carrier. A blind passage, consisting of transfer by inoculation of normal organ extracts from one host to another, even within the same inbred line, may sufficiently increase its pathogenic properties to cause development of parotid tumors. Under normal conditions of life, however, the parotid tumor agent would not become pathogenic spontaneously, except on very rare occasions (we have seen only one untreated C3H mouse that developed parotid tumor spontaneously)(6). It is therefore possible that the parotid tumor agent is essentially of low pathogenic potency for its natural carrier. Its presence in mice of various inbred strains would manifest itself only exceptionally under normal conditions, although this agent may be present in a number of inbred strains of mice. Spontaneous development of parotid tumors is apparently

TABLE III. Results of Inoculation of Centrifuged Extracts from Fresh, Lyophilized, or Glycerinated Normal C3H or C3H(f) Organs into Newborn (<15 Hr Old) C3H or C3H(f) Mice.

No. of exps.	Material	Strain	No. of mice Inoc.	Dev. parot. tumors
4	Fresh		24	2
5	Lyophil. preserv. at −4°C, ½ to 26 mo		37	5
2	Glycer. preserv. at −4°C, 1½ to 4½ mo		13	3
11		C3H	53	8
		C3H(f)	21	2
		Total	74	10*

* Avg age parot. tumors developed 4 mo.

exceptional even in descendants of mice bearing such tumors. We bred C3H males and females with induced parotid tumors, and raised 61 offspring of such tumor-bearing parents by brother-sister mating, through 4 successive generations, but none developed either leukemia or parotid tumors(9).

There may exist oncogenic agents not pathogenic for their natural carrier-hosts. This could be compared with the "hereditary" transmission of certain phages, which may be non-inducible in certain bacterial carriers, but may be readily induced in susceptible detector-strains(10). The parotid tumor agent may be similar.

The leukemic agent also carried and transmitted from one generation to another in mice of Ak and C58 inbred lines, is highly pathogenic for its carriers, causing the development of "spontaneous" leukemia in 90% of its natural hosts. In this case, no "blind transfer" is needed to induce the disease. Moreover, activation of the latent leukemic agent can be prompted in the natural carrier by various means, such as hormones, radiation energy, or carcinogens, acting on a receptive host. The leukemic and parotid tumor agents would therefore differ fundamentally in their biological and pathogenic properties. Whether their association in the high leukemic Ak and C58 inbred lines is coincidental, is yet to be determined.

Development of soft tissue sarcomas among some of inoculated C3H mice is quite interesting. These tumors (Fig. 2 and 3) resembling fibro-sarcomas, myxosarcomas, and occasionally rhabdomyosarcomas with multinuclear cells, developed in most instances subcutaneously. In some instances sarcoma developed in muscles, or in muscular or perineural fascia, and in 2 instances in the uterus (rhabdomyosarcoma). We have never observed similar tumors developing spontaneously in untreated C3H mice. These tumors could be induced occasionally by inoculating newborn C3H mice with centrifuged or filtered Ak leukemic extracts, more frequently with cell-free extracts prepared from induced C3H leukemias or from induced parotid tumors, and also with extracts prepared from normal C3H mammary glands. They were never observed in C3H mice which developed induced leukemia, but some appeared in animals that developed also parotid tumors. Only a few of these sarcomas could be transplanted, by cell transfer, in C3H mice of the same substrain. When transplanted successfully, they developed at the site of implantation within a few weeks, growing then locally. An attempt to recover an agent from such sarcomas is now in progress(9). These *sarcomas are so different from leukemic or parotid tumors,* that it is difficult to assume that they might be caused by the same agent. If caused by a distinct oncogenic agent, it appears that the sarcoma agent would also be usually not pathogenic for its natural carrier. Thus, neither the Ak, nor the C3H mice, even though potentially able to induce sarcomas, develop these tumors spontaneously. As with the parotid tumor agent, following blind passage into a newborn, susceptible host, this agent may become sufficiently pathogenic to induce sarcomas. A potent inducing factor, such as methylcholanthrene, could also activate this masked agent, causing development of a sarcoma of similar morphology in the natural carrier(11).

The possibility that a single host may carry several distinct oncogenic viruses, in masked form, may appear remote. However, the number of bacterial and filterable viruses, many potentially pathogenic, carried by animals and human hosts, is so large, that oncogenic agents would represent only a small addition to this collection of parasites and symbionts. Carried by apparently healthy hosts, and transmitted from one generation to another, these agents would cause only occasionally symptoms of disease, remaining in most instances harmless for their natural carrier-hosts(12).

Summary. 1. Parotid tumors or leukemia developed following inoculation of Ak extracts into newborn C3H mice. When extracts from induced C3H leukemias were inoculated into 105 newborn C3H mice (Bittner substrain), 25 (24%) developed leukemia, 12 (11%) parotid tumors, and 7 (7%) subcutaneous sarcomas. Of 296 newborn C3H mice inoculated with parotid tumor extracts, 24 (8%) developed leukemia, 21 (7%) parotid tumors, 15 (5%) subcutaneous sarcomas, 2 submaxillary gland tumors, and 2 medullary adrenal tumors. 2. Centrifuged extracts from normal C3H embryos were inoculated into 103 newborn C3H mice, and 3 developed parotid tumors. Of 9 newborn C3H mice inoculated with C57 Brown/cd embryo extracts, 1 developed parotid tumor. When centrifuged extracts prepared from normal C3H mammary glands were inoculated into 60 newborn C3H mice, 9 developed parotid tumors, and 5 subcutaneous sarcomas. Centrifuged pooled normal C3H(f) and C3H organ (liver, spleen, heart, kidney) extracts fresh, or preserved either by dry-freezing or in 50% glycerine, were inoculated into 74 newborn C3H mice, and 10 (14%) developed parotid tumors. 3. Physiological saline solution or sterile tryptophane broth inoculated into 62 newborn C3H mice, did not induce tumors or leukemia. 4. Ak mice may carry not only a leukemic, but also another oncogenic agent, the latter not pathogenic for its natural carrier. C3H mice may also carry such a masked agent. A blind transfer into a newborn susceptible (C3H) host may sufficiently increase its pathogenic potency to cause parotid tumors. The sarcomas may be caused by another, individually distinct, though biologically similar agent.

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Received February 2, 1955. P.S.E.B.M., 1955, v88.

Rapid Virulence Test in Diagnosis of Diphtheria. (21590)

E. I. PARSONS, M. FROBISHER, M. MOORE, AND M. A. AIKEN.*

From the Communicable Disease Center, Public Health Service, U. S. Department of Health, Education, and Welfare, Atlanta, Ga.

The accuracy, sensitivity, and practical value of the *in vitro* toxigenicity (virulence) test (I.V.T.T.) for pure cultures of *C. diphtheriae* are now well established(1-3). In this method one inoculates the pure culture to be tested in a straight line on the surface of Petri plates containing a special serum agar (3) and a centrally placed source from which diphtheria antitoxin diffuses into the agar. Toxin diffuses from the inoculum and antitoxin diffuses from the central source. Where the two meet in the agar, in proper proportions, a visible white precipitate forms. This constitutes a positive toxigenicity test.

Heretofore isolation of pure cultures of the test organism, with consequent delay of 72 to 96 hours, has been considered necessary to a proper I.V.T.T. Recent studies by Santopadre(4) in Italy and by the authors, show that it is possible, by means of the I.V.T.T., to demonstrate the presence of toxigenic *C. diphtheriae* in original diagnostic material 24 hours after receipt of the specimen. The special medium for the I.V.T.T. is made somewhat selective by adding to it 0.0045% of potassium tellurite, which retards growth of many of the contaminating organisms in the original throat swabs or Loeffler cultures but has little inhibitory effect on Corynebacteria. The plates are inoculated from the original throat cultures or with the original swab, and incubated and read in the same manner as for testing toxigenicity of pure cultures of *C. diphtheriae*.

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* Present address: Department of Bacteriology, University of Kentucky, Lexington.

Methods, (1). In the present series 266 throat cultures on Loeffler slants, all of which had been incubated at least 18 hours and subsequently held at room and/or icebox temperature for 4 days to 6 weeks, were each inoculated directly onto I.V.T.T. plates. Precipitates characteristic of toxigenic *C. diphtheriae* were visible within 24 hours in 63 of the 266 cultures; in 48 hours in 53; and in 72 hours in 14; 130 in all. Parallel platings on cystine-tellurite-blood plates yielded pure cultures of *C. diphtheriae* from 205 of the cultures. Of these, 9 were atoxigenic when tested separately both on I.V.T.T. plates and in animals. These atoxigenic cultures came from Loeffler slants which had yielded negative results in the mixed-growth I.V.T.T. Thus 130 (66.3%) of 196 original throat cultures, later shown to contain toxigenic *C. diphtheriae* by pure culture isolation, were demonstrated to contain toxigenic *C. diphtheriae* 24-72 hours after arrival in the laboratory.

Methods, (2). Although with reasonable care in performance of the I.V.T.T. false positives and negatives rarely occur, it was thought desirable to submit a representative group of the organisms isolated in this series to test in animals. Pure strains were isolated from all of the Loeffler slants which had given positive I.V.T.T. Seventy-two of these were selected at random and tested in rabbits by a standard procedure(5). All gave clearly positive results. Nineteen pure strains from Loeffler slants negative by I.V.T.T., similarly tested, gave negative results. The same pure cultures, positive and negative, tested by I.V.T.T., all gave concordant results. There is, therefore, no reason to think that the use of mixed cultures increases the risk of introducing false positives into the test. However, in this, as in many other diagnostic procedures, a negative reaction obviously does not exclude the possibility that a specimen might yield a positive result under other test conditions.

Methods, (3). Since Loeffler cultures usually incubate overnight before streaking on the I.V.T.T. plates an attempt was made to obtain positive toxigenicity tests directly from the original throat swabs themselves. As stated before, the material tested in this study consisted of old Loeffler cultures since this laboratory does not commonly receive original swabs. Eighteen "artificial" throat swabs were prepared by mixing normal throat flora from fresh, negative, Loeffler slants with small amounts of diluted pure culture of toxigenic *C. diphtheriae* and moistening the swabs with the mixtures. Stained smears from these swabs showed diphtheria bacilli to be present in relatively small numbers among large numbers of other bacteria. After standing for 24 hours, these swabs were streaked directly on I.V.T.T. plates. Nine yielded positive reactions in 24 hours; 7 others were positive in 48 to 72 hours. Two failed to give a positive result. In all, about 89% gave a positive I.V.T.T. directly from the swabs.

Summary. The mixed growth from 266 original Loeffler slants submitted for diagnosis of suspected diphtheria was streaked directly onto tellurite-agar plates prepared for the *in vitro* toxigenicity test (I.V.T.T.). Of 196 such diagnostic specimens later shown, by pure culture isolations and animal tests, to contain virulent *C. diphtheriae,* 130 (66.3%) yielded positive results; 63 (48%) in 24 hours, 53 (40%) in 48 hours and 14 (11%) in 72 hours. Nine of the original 266 cultures were later shown by pure culture isolations and animal tests to contain non-toxigenic *C. diphtheriae*. These yielded negative I.V.T.T. Preliminary tests with prepared swabs indicate that positive I.V.T.T. frequently may be obtained directly from them in 24 hours. The I.V.T.T. plate, with K Te, offers a rapid and simple method of detecting virulent *C. diphtheriae* in a useful percentage of original diagnostic specimens.

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Received February 4, 1955. P.S.E.B.M., 1955, v88.

Hemagglutination Behavior of Mumps Virus Strains of Different Origin.* (21591)

VICTOR J. CABASSO AND HERALD R. COX.
From Viral and Rickettsial Section, American Cyanamid Co., Lederle Laboratories, Pearl River, N. Y.

The demonstration of a specific mumps virus hemagglutinin in infected chick embryo fluids by Levens and Enders(1) and Beveridge and co-workers(2) has resulted in practical laboratory diagnostic procedures for the detection and titration of the virus and for the measurement of mumps antibodies(3). Although erythrocytes from different species of animals were found to be susceptible to hemagglutination by mumps virus(4), chicken and human Group O cells have been the most routinely used for both diagnostic and experimental testing(3). It has also been a general practice to carry out the hemagglutination test at room temperature without due consideration to the variations in temperature which may occur in the same laboratory, from one laboratory to another and from country to country.

The object of this paper is to report some differences observed between the hemagglutinating capacity of mumps virus strains of different origins at various temperatures, using erythrocytes derived from chickens, guinea pigs and human Group O.

Materials and Methods. Origin of virus strains.† Strain E was received from Dr. Enders' laboratory in its 37th chick embryo passage; the Ha strain was obtained from Dr. Habel at the 20th passage level; strain He, a side-line of the E strain, which had undergone 16 allantoic cavity passages, was received from Dr. G. Henle; and strain Ja was isolated here from the spinal fluid of a mumps patient. The first chick embryo passage of this latter strain was made in the amniotic sac, and this was followed by three more consecutive passages in the allantoic cavity. *Preparation of erythrocytes.* The erythrocytes used consisted of pooled cells derived from at least three or four chickens, guinea pigs or human Group O individuals. The citrated blood pools were washed in normal saline three times, centrifugation being carried out between washings at 1,000 rpm for 10 minutes in an anglehead centrifuge. A final centrifugation at 1,500 rpm for 15 minutes was used for packing. The washed, packed erythrocytes were made into a 30% suspension in normal saline and stored at 4°C for not more than five days. Aliquots of this suspension were diluted as desired just before the performance of the tests. *Titration method.* All titrations were made in saline by the Salk(5) hemagglutination procedure, using 0.25% suspensions of the different erythrocytes. Triplicate titrations of each preparation were made with each cell species and placed at 4°C, room temperature (22-24°C) and 37°C, respectively. Because of the slower rate of sedimentation of guinea pig and human erythrocytes, especially at 4°C, readings were made after two hours incubation at the various temperatures.

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* The authors wish to express their appreciation to Mrs. Eleanor Osborn for assistance in preparation of the manuscript.

† The authors wish to express their thanks to Drs. Enders, Habel and G. Henle for strains of virus mentioned above.

TABLE I. Titration of 2 Strains of Mumps Virus in Presence of Human O, Chicken and Guinea Pig Erythrocytes of 3 Different Temperatures.

Virus strain	Erythrocyte	Temp., °C	Allantoic fluid dilutions 1:20	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560	Erythrocyte saline control
E (43)*	Human O	4	+	+	+	+	+	±	—	—	—
		22-24†	+	+	+	+	+	±	—	—	—
		37	Gr	Gr	+	+	+	±	—	—	—
	Chicken	4	+	+	+	+	+	+	—	—	—
		22-24	+	+	+	+	+	+	±	—	—
		37	Gr	Gr	Gr	+	+	+	—	—	—
	Guinea pig	4	+	+	+	+	+	±	—	—	—
		22-24	+	+	+	+	+	±	—	—	—
		37	Gr	Gr	+	+	+	+	±	—	—
Ha (25)	Human O	4	—	—	—	—	—	—	—	—	—
		22-24	—	—	—	—	—	—	—	—	—
		37	—	—	—	—	—	—	—	—	—
	Chicken	4	+	+	+	+	+	±	—	—	—
		22-24	+	+	+	+	+	±	—	—	—
		37	—	—	—	—	—	—	—	—	—
	Guinea pig	4	+	+	+	+	±	—	—	—	—
		22-24	+	+	+	+	±	—	—	—	—
		37	+	+	+	+	—	—	—	—	—

* Chick embryo passage level used in test. † 22-24°C = Room temperature.
Gr = Granular aspect of cellular aggregate.

The results given in Table I are typical of those obtained on a great number of titrations of the E and Ha strains of virus. Whereas the E strain was capable of agglutinating all 3 types of cells used at all 3 temperatures, human O cells completely failed to reveal the presence of strain Ha at the same temperatures; furthermore, no hemagglutination occurred in the presence of chicken cells with titration of strain Ha at 37°C. It is noteworthy that, although strain Ha titers in the presence of guinea pig cells were somewhat lower than those obtained with chicken cells, detection of the virus was obtained at all 3 temperatures. It seemed, therefore, that each of the 2 strains tested possessed a definite hemagglutinating pattern, which could be used to distinguish one from the other. In view of these findings, the behavior of the other strains of virus given under Materials and Methods was investigated by the same technic. The results of repeated titrations are summarized in Table II. It was not surprising to obtain with strain He a pattern identical with that of strain E because of their common origin. A similar pattern was also obtained with strain Ja. The virus preparation designated as unknown in Table II consisted of a concentrated mumps virus antigen of either the E or Ha strains, prepared several months before these tests. Through an oversight, the name of the strain with which it was prepared had been omitted from the label. However, the pattern obtained with this antigen clearly indicates that it was prepared with strain E.

Discussion. From the standpoint of their antigenic structure(4) as well as their reaction to physical and chemical agents, mumps virus strains isolated from distant parts of the globe have been found to be similar, if not identical. It is, therefore, of interest that some differences should be found in their hemagglutination behavior in the presence of cells of different species. The most unexpected feature of these differences is the fact that human Group O cells should be found to be the least susceptible to agglutination by a strain of mumps virus, a host-specific parasite of man. The question is whether these differences constitute intrinsic characteristics of the virus strains studied, or whether they have evolved as a result of their different passage histories in the chick embryo. Should the first of the two explanations be accepted, it stands to reason that the presence of a

TABLE II. HA Pattern of Several Mumps Virus Preparations in Presence of Human O, Chicken and Guinea Pig Erythrocytes at 3 Different Temperatures.

	Erythrocyte and temperature								
Virus preparations	Human O			Chicken			Guinea pig		
	4°C	22-24°C*	37°C	4°C	22-24°C	37°C	4°C	22-24°C	37°C
E (43)†	+	+	+ Gr	+	+	+ Gr	+	+	+ Gr
Ha (25)	—	—	—	+	+	—	+	+	+
Ja (4)	+	+	+ Gr	+	+	+ Gr	+	+	+ Gr
He (19)	+	+	+ Gr	+	+	+ Gr	+	+	+ Gr
Unknown	+	+	+ Gr	+	+	+ Gr	+	+	+ Gr

* 22-24°C = Room temperature. † Chick embryo passage level used in test.
Gr = Granular aspect of cellular aggregate.

strain of virus similar to Ha could be missed if only human Group O cells were used. Similarly, a strain of the same type would also pass unnoticed in the presence of chicken cells, should the environmental temperature be close to 37°C. Rapid loss of hemagglutination of chicken cells by strain Ha at 37°C may be attributed in part to an extremely rapid rate of elution of the virus and to the relative lability of the hemagglutinin of this strain of virus at 37°C, as previously reported(6).

Under the conditions of the experiment above, it seems that guinea pig cells may be a most reliable reagent for the detection of mumps virus, within a wide temperature range. When using these cells in the hemagglutination-inhibition test, however, Beveridge and Lind(4) have found it necessary to absorb the serum under test with guinea pig cells in order to remove natural hemagglutinin for erythrocytes of this species.

Summary. Differences in hemagglutination behavior have been found to exist between mumps virus strains of different origins. Most strains tested were capable of hemagglutinating chicken, guinea pig and human Group O cells at 4°C, 22-24°C and 37°C. One strain did not agglutinate human Group O cells at any of these temperatures, and failed also to agglutinate chicken cells at 37°C. However, this same strain was able to cause hemagglutination of guinea pig cells at all 3 temperatures. The implications of these findings in relation to laboratory testing for mumps virus has been discussed.

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Received February 4, 1955. P.S.E.B.M., 1955, v88.

Characteristics of a Thermophilic Bacteriophage.* (21592)

Roberta White, Carl E. Georgi, and Walter E. Militzer.
From Departments of Bacteriology and Chemistry, University of Nebraska, Lincoln.

Most microorganisms are susceptible to infection by bacteriophage and probably every thoroughly investigated group will eventually be found to be host to some phage. Many of the bacteria which are known to be infected by viruses are mesophilic organisms such as enteric bacteria, bacilli, staphylococci, streptococci and corynebacteria. A few instances of thermophilic bacteriophage have been recorded. Koser(1) isolated a thermophilic phage and observed the optimum temperature for lytic activity of the virus to be about 50°C. It was capable of lysing the host at 57°C to 58°C, survived heating at 70°C for 30 minutes but was completely inactivated at 75°C. Adant(2) reported a lytic principle which developed readily at 52°C to 55°C but did not survive heating for 30 minutes at 75°C.

Recently a bacteriophage, capable of lysing a thermophilic bacterium at 65°C, was isolated in this laboratory from green house soil, and studied to determine its morphology, host range, temperature range, plaque morphology, heat resistance in various suspending media, burst size and latent period.

Materials and methods. Organisms serving as hosts for phage were aerobic, sporogenous bacilli designated NCA 2184, originally recovered from spoiled canned peas, and No. 4 and No. 10 isolated from thermal springs in Yellowstone National Park, all characterized by Marsh and Larsen(3) as being or resembling closely *Bacillus stearothermophilus*. The bacteria were grown in broth containing 2.0% Trypticase, and on solid media containing 0.0007% $FeCl_3$, 0.0015% $MgSO_4$ and 2.0% agar in addition to Trypticase. The agar layer method described by Swanstrom and Adams(4) was employed to obtain high titre phage stock. Phage lysates were stored 5 to 7 days before purification, then centrifuged at 4000 rpm for 20 minutes to remove agar and larger particles. After filtration through either a Berkefeld or sintered glass filter, the lysate was placed in sterile steel cups and centrifuged at 25,000 G for 1 to 2 hours. The agar layer method of titration was used throughout for determining the number of infectious units per ml. Because of the high temperature of incubation, 2% agar was used in the lower layer and a 0.9 to 1.0% agar in the soft layer. *Adsorption* studies were carried out by singly infecting host suspensions which were in the logarithmic growth phase. At intervals, a 0.1 ml aliquot was removed and diluted 100 fold to stop adsorption. After centrifuging the mixture at 4000 rpm for 10 minutes, to remove the bacteria containing adsorbed phage, the supernatant was assayed for free phage by the agar layer technic. The *one-step growth curve* method described by Ellis and Delbrück(5) which combines the determination of both burst size and latent period and a modification suggested by Adams(6) were employed to determine these characteristics. *Preparations of concentrated phage* for electron microscopy were made according to the method of Labaw and Mosley (7). Formalin was added to the filtered lysate to yield a 2% concentration and allowed to stand for 24 hours. After fixation, the lysate was ultracentrifuged at 25,000 G for one hour, the sediment was taken up in 15 ml of saline and centrifuged at 4,000 rpm to remove large particles. Following ultracentrifugation of the saline suspension, the sediment was taken up in about 1 ml of distilled water and again cleared by low speed centrifugation. The suspension was prepared by infecting the host cells in a ratio of 10 virus particles for every bacterium. At intervals, a small amount was withdrawn with a capillary pipette and applied to the grid. *Heat inactivation* studies were performed as reported earlier by White, Georgi and Militzer (8).

Results. Plaques obtained at the initial

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* This investigation was supported in part by a research grant from the National Microbiological Institute of the National Institutes of Health, Public Health Service.

isolation exhibited 3 main types of morphology: small ones with a clear halo about 1 mm in diameter, large 3 to 4 mm plaques with a clear halo, and large ones with a turbid halo. Large plaques with a clear halo were picked for purification. It has been observed that the growth of certain phages was inhibited by the presence of citrate ion in the culture medium and that this inhibition could be overcome by the addition of calcium salts (Adams)(9,10) since the antagonistic effect of citrate results from the removal of calcium and magnesium by complex formation. Friedman and Cowles(11) added sodium citrate to hard agar and semisolid agar in two different concentrations and found that the M series of *Bacillus megaterium* phages were inhibited in different degrees in the presence of citrate. When 0.2% sodium citrate was added to hard and semi-solid Trypticase agar, the thermophilic phage failed to produce any plaques on solid media whereas they were quickly formed on agar free of citrate. Stock phage of sufficiently high titre could not be obtained in broth lysates, the highest number of infectious particles produced was 10^8 per ml. The agar layer method, however, yielded titres of about 10^{10}.

Twenty-seven thermophilic bacilli from the stock culture collection at the University of Nebraska were tested for susceptibility to the newly isolated virus. Of this group NCA 2184, No. 10 and No. 4 were lysed by the virus. Several mesophilic spore-forming bacilli were also exposed to the phage at temperatures of 37°C and 45°C. No lytic activity could be observed at either temperature, indicating that the host range did not cross the boundaries from thermophilic to mesophilic.

Phage suspensions of the same titre were plated and incubated at various temperatures to determine the optimum for maximum lytic activity as manifested by the number of plaques formed. This number was greatest at 65°C, fewer appeared at 70°C, 55°C and 50°C; there were no plaques at 40°C.

Adsorption of virus to bacterium is influenced by factors such as concentration of electrolytes and activation by amino acid cofactors. Electrolytes, in proper proportions, enhance adsorption but excessive amounts may hinder the process, the inhibitory effect being greater at higher temperatures (Fong (12)). Delbrück(13), studying the co-factor requirements for adsorption of the T series of coli-dysentery phages, demonstrated that some viruses require tryptophane or similar substances and, in addition, calcium ions.

The length of time required for the virus to adsorb on to the 3 hosts was determined in 2% Trypticase broth and in 2% Trypticase broth containing either 0.01% $CaCl_2$ or 0.01 % $MgSO_4$. About 75% of the phage was adsorbed onto No. 4 within 30 minutes, 84% on No. 10 and 65% on No. 2184 when suspended in Trypticase broth. In broth containing the $MgSO_4$ only 14% of the phage was adsorbed on to No. 4 whereas 85% was adsorbed in 30 minutes in broth containing the $CaCl_2$. Less phage was adsorbed in broth containing higher than 0.01% concentration of $CaCl_2$.

The one-step growth curve did not yield the information desired. Because of the slow adsorption of the virus and the continued growth of the infected host cells, a continued rise in the number of phage particles occurred instead of a leveling off after all the bacterial cells had lysed. The average burst size could not be determined. The latent period was roughly estimated to be about 80 to 90 minutes after adsorption of 85% of the virus.

Most viruses, in crude preparations or in media of complex organic composition, are quite stable at room temperature. At temperatures of 50°C to 60°C, inactivation often becomes measurable for this is within the range where protein denaturation occurs at a considerable rate (Luria)(14).

Nanavutty(15) studied the rate of inactivation of a coli-dysentery phage exposed to various temperatures in different suspending media and found that susceptibility to heat inactivation was dependent upon the nature of the substrate. Krueger(16) demonstrated with staphylococcus phage that inactivation of bacterial viruses follows first-order kinetics and that the temperature coefficient is of a magnitude characteristic of protein denaturation. Koser(1,17) reported that a thermophilic phage, whose optimum temperature for

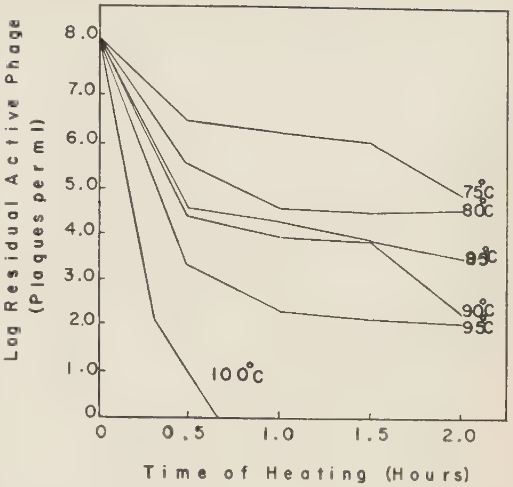

FIG. 1. Heat inactivation of the thermophilic phage suspended in 0.5% gelatin.

lytic activity was 55°C, did not require a correspondingly higher temperature for its destruction. Tests showed that it resisted temperatures up to, and including 70°C, but was destroyed at 75°C when exposed for 30 minutes. Adant(2) isolated a thermophilic phage which also was destroyed at 75°C in 30 minutes.

Heat inactivation studies of the newly isolated phage were carried out in 4 different suspending media and exposed to temperatures ranging from 75°C to 100°C to determine whether it might be more resistant to heat than phages thus far reported. Previous studies (White, Georgi and Militzer(8)) on the phage lysate revealed that the phage was not inactivated at a constant rate and that some particles survived heating at 95°C for 2 hours. When the logarithm of residual active phage was plotted against time, it was observed that straight line curves were not obtained indicating that inactivation did not follow a unimolecular reaction. When exposed to 75°C for 2 hours, 3.4 x 10^5 phage particles capable of producing plaques survived as compared to 10^8 per ml at 70°C. Two hours exposure of the lysate at 80°C reduced the number to 13,000 plaques. At 100°C there were but 50 plaques per ml detectable following 30 minutes of exposure with complete inactivation at the end of an additional half hour. Regardless of the temperature, most rapid inactivation occurred during the first half-hour after which the rate decreased quite markedly. Plaque-producing phage particles observed at 0.5 hour were 35,000 at 85°C, 6,000 at 95°C whereas at the end of 2 hours there still remained 3,500 and 1,000 particles per ml respectively. Experiments designed to demonstrate whether the heat-surviving phage was hereditarily more heat resistant showed that the former were not more resistant than the original phage from which it was selected.

Phage suspended in 0.5% gelatin at pH 7.0 responded to heat inactivation in a manner very similar to the crude lysate suspension (Fig. 1). Again the curves were concave, most rapid inactivation occurring during the first half hour, the rate decreasing after this time. After 2 hours at 90°C there were 200 particles remaining. At 95°C the numbers remaining in gelatin were fewer at each time interval than remained in the crude lysate at corresponding times. Phage suspended in phosphate buffer at pH 7.0 (Fig. 2) did not survive exposure to high temperatures as it did in its own lysate or in the presence of gelatin. At 75°C about 6,500 phage particles remained in buffer at the end of one hour as compared to 3.0 x 10^6 at the same period in the lysate. The virus exhibited no activity after one hour at 80°C whereas 37,000 particles remained in the lysate at the end of 60

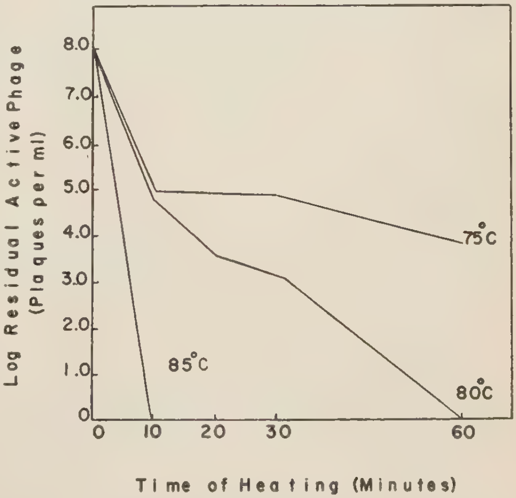

FIG. 2. Heat inactivation of thermophilic phage suspended in phosphate buffer.

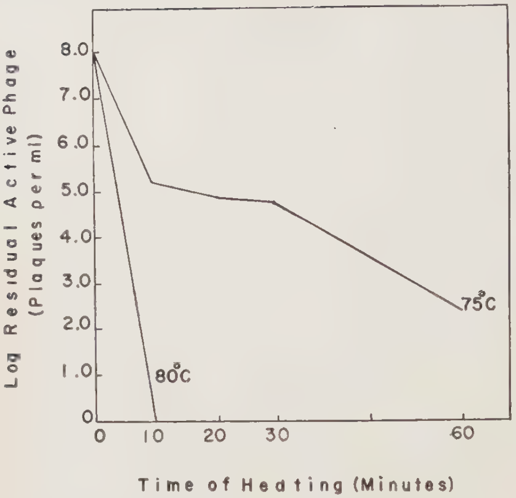

FIG. 3. Heat inactivation of thermophilic phage suspended in distilled water.

minutes. Ten minutes exposure at 85°C completely inactivated the virus.

When the phage was suspended in distilled water, it was inactivated most rapidly; only 220 particles remained at the end of 60 minutes when exposed to 75°C. At 80°C, only 10 minutes were required to completely inactivate the phage (Fig. 3). The hydrogen ion concentration, after addition of the phage, was between pH 6.5-6.7.

Electron micrographs of the purified concentrated phage could be obtained only when the lysate was fixed in 2% formalin before subjecting it to centrifugation. Fig. 4 shows the thermophilic phage which is of the tailed variety. Specimens not fixed in formalin prior to centrifugation showed only flattened, round heads without tails.

Discussion. A bacteriophage was isolated from green house soil, which like other bacterial viruses was specific in that it lysed only 3 of the 27 thermophilic spore-forming bacilli tested. Mesophilic, Gram-positive spore-forming rods were not susceptible to the action of the phage.

Few plaques were produced at temperatures of 55°C or below, but much larger numbers were formed at 65°C, indicating that the latter was more nearly optimum for lytic activity. Thermophilic organisms are defined as those which develop best at temperatures above 45°C to 55°C, in contrast to the thermoduric organisms which only survive exposure to high temperatures. The newly isolated phage meets these conditions and therefore it can be said that it is truly thermophilic and not thermoduric.

The amount of phage and the rate at which it is adsorbed is very slow when compared to mesophilic phages. Calcium chloride enhances its adsorption, increasing the amount taken up in 30 minutes by 15 to 20%; magnesium sulfate permitted very little adsorption on isolate No. 4.

One-step growth curve experiments and burst size determinations were not made because of the very slow adsorption of phage by the host.

It can be concluded from the data in Fig.

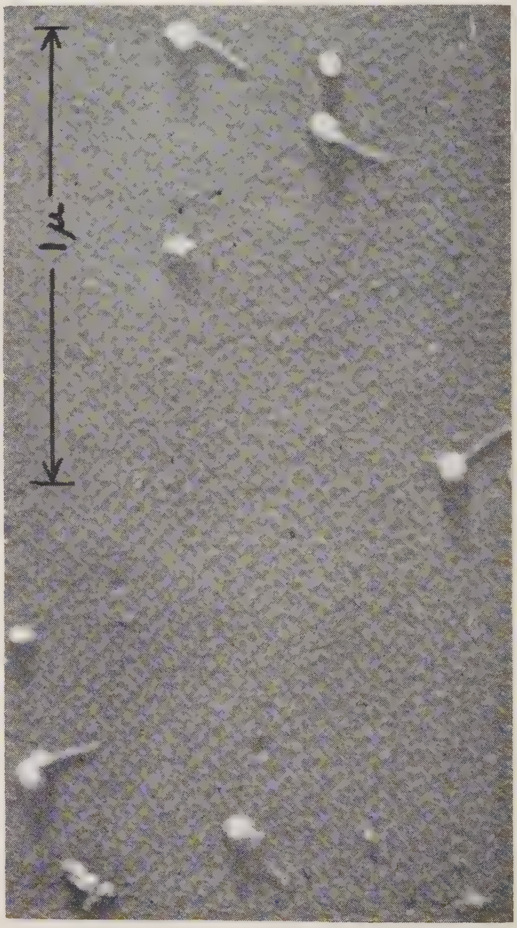

FIG. 4. Electronmicrograph of thermophilic phage, formalin fixed, uranium shadowed.

2 and 3 that the phage suspended in phosphate buffer (pH 7.0) and in distilled water exhibited much less resistance to heat than when suspended in either broth lysate or gelatin. Phosphate buffer afforded somewhat more protection than did distilled water. When the logarithm of residual active phage was plotted against time for the phage suspended in gelatin it was found that the curves were strikingly similar to those obtained in crude lysate. The foreign protein evidently affords the virus protection against high temperatures. These findings are in accord with the heat susceptibility properties of the reported mesophilic phages.

These data bear out the hypothesis that the virus used in this investigation is more heat resistant than any previously reported (Koser (1,17), Adant(2)).

Summary. 1. A new thermophilic phage host system has been studied and described. It has been designated a thermophilic phage because the greatest number of infectious units was produced at 65°C, above or below which the number of particles was considerably less. 2. Phage is adsorbed very slowly, the maximum amount taken up being 85% in 30 minutes. 3. Thermal inactivation studies indicate that inactivation does not follow a monomolecular reaction but the rate decreases with time. This bacteriophage is not as vulnerable to heat when suspended in lysate or gelatin as it is in distilled water or phosphate buffer and is much more resistant to heat than other previously reported mesophilic or thermophilic phages.

We wish to express our appreciation to Dr. Thomas L. Thompson for his advice on some phases of the investigation and to Prof. Wendall Robison of the University Research Council's Instrumentation Laboratory for assistance in taking the electron micrograph.

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Received February 7, 1955. P.S.E.B.M., 1955, v88.

Electron Microscopic Morphology of Frozen-Dried Particles of Meningo-Pneumonitis Virus.* (21593)

T. Timothy Crocker and Robley C. Williams. (Introduced by W. M. Stanley.)

From Department of Medicine, and George Williams Hooper Foundation for Medical Research, University of California Medical Center, San Francisco, and Virus Laboratory, University of California, Berkeley.

Morphological observations and measurements of the psittacosis-lymphogranuloma venereum (P-LV) group of viruses have been made in the past by electron microscopy of air-dried specimens (dried in air from aqueous suspending media). In such preparations there is universally seen in electron micrographs a central electron-dense elevation, surrounded by a less dense flattened periphery of material resembling a membrane. The shape of the air-dried particles implies that, when hydrated, they are probably spheres consisting of a central mass surrounded by a more fluid material possessing membrane-like limits.

To obtain evidence as to whether or not the particles of the P-LV group of viruses are indeed spheres when in aqueous suspension, the freeze-drying method(1) of mounting suspended particles may be advantageously used. This method has been shown to preserve the shape of hydrated particles by rapid freezing; during subsequent removal of water by sublimation of the ice at low temperature, the particles retain the three-dimensional shape which they had at the instant of freezing.

Methods. The material employed for the studies reported here was allantoic fluid from chick embryos infected with meningo-pneumonitis (M-P) virus, a member of the P-LV group. The embryos were infected and the fluid withdrawn by methods described previously(2). Virus particles were concentrated by centrifugation and separated from contaminating normal tissue components by brief treatment with sodium desoxycholate and freed of salt by dialysis as described elsewhere(3). The particulate suspension was sprayed upon collodion membranes maintained at about –80°C; a portion was also mounted on collodion and air-dried in the usual manner. Polystyrene latex spheres, 260 mμ diameter, were included in each preparation to demonstrate the relationship of shadow length to height for typical spheres and to provide an internal standard of magnification.

Results. An air-dried elementary body of M-P virus is shown in Fig. 1. It is evident that the particles have flattened upon drying, yielding the "derby hat" appearance described by Rake *et al.*(4) in the case of feline pneumonitis virus. That this characteristic appearance of air-dried viruses of the P-LV group is misleading is demonstrated in Fig. 2, which is an electron micrograph of some frozen-dried M-P virus elementary bodies.

The electron-dense central mass of the frozen-dried elementary bodies, (*2*) of Fig. 2, is bounded by a delicately wrinkled periphery of low electron-opacity, and the entire particle casts a shadow similar in length and shape to that cast by the latex sphere (*1*) in the same field. The diameter and height of the elementary bodies are equal, indicating that there has been no detectable flattening of the particle during the removal of the water vapor. Particles from which the dense central material has been released form sac-like ghosts of low electron density, (*2G*) of Fig. 2. The empty particles do not regularly appear spherical, and are either not spherical in suspension or do not retain their original spherical shape when dried. It is likely that the freeze-dry method does not provide perfect retention of the original shape of hydrated particles which have little intrinsic rigidity.

Particles of the type shown in Fig. 2 (*2*) are 250 to 270 mμ in diameter, while similar particles prepared by air drying measure 380 to 435 mμ in diameter. The diameter of hydrated particles of the P-LV group has been reported to be 0.22 to 0.33 μ(5) and 200 to

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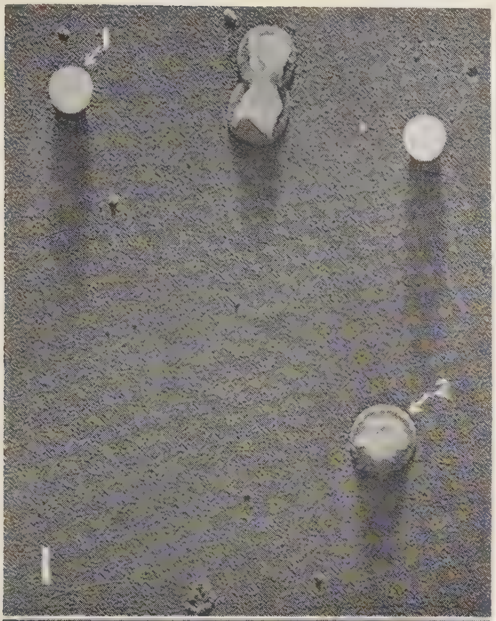
* This work supported by Contract between Veterans Administration and University of California, and American Cancer Society upon recommendation of Committee on Growth of the National Research Council.



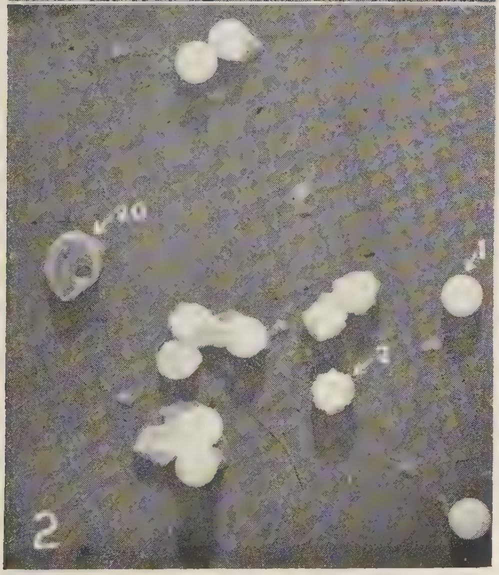


FIG. 1. Air-dried preparation containing polystyrene latex spheres (1) and elementary bodies of meningo-pneumonitis virus (2) in distilled water.

FIG. 2. Frozen-dried preparation containing polystyrene latex spheres (1), elementary bodies (2) and an elementary body "ghost" (2G) of meningo-pneumonitis virus in distilled water.

300 mμ(6,7) by collodion membrane filtration. Analytic centrifugation of homogenous suspensions has furnished estimates of 250 mμ for the diameter of the elementary bodies (8). These values for the diameter of the hydrated particles are in good correspondence with the measurements of the frozen-dried elementary bodies, indicating that the freeze-drying method preserves the shape and dimensions of the virus particles in close approximation to the form in which they exist in suspension. Since the virus particles contained within infected cells are probably spherical, diameter measurements of virus particles in sectioned cells may be expected to correspond more closely with those obtained from frozen-dried preparations than from air-dried specimens. The latter interpretation would seem to be confirmed by electron microscopy of thin sections of infected chorio-allantoic membranes by Gaylord(9) and in cells from infected tissue cultures examined by us and to be reported later.

Summary. Preparation of elementary bodies of meningo-pneumonitis virus for electron microscopy by freeze-drying provided spherical particles of 250 to 270 mμ diameter instead of the flattened particles of 380 to 435 mμ diameter obtained by mounting and air drying the specimen in the usual manner. The diameters of the frozen-dried particles corresponded well with diameters estimated by filtration and centrifugation of elementary bodies in the suspended state. Thus, the freeze-dry method preserved approximately the form and dimensions which the elementary bodies possessed in the hydrated state and probably in the infected cell.

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Received February 9, 1955. P.S.E.B.M., 1955, v88.

Ammonia Levels in Blood and Cerebrospinal Fluid.* (21594)

William V. McDermott, Jr., Raymond D. Adams, and Athol G. Riddell.†
(Introduced by J. C. Aub.)

From Departments of Surgery and Neurology, Harvard Medical School and the Surgical and Neurological Services, Massachusetts General Hospital.

In recent months there has been a reawakening of interest in the metabolism of ammonia. Studies from this laboratory have emphasized that elevations in blood ammonia levels are invariably associated with "meat intoxication" in the Eck fistula dog(1) and with the episodes of stupor following the surgical construction of portacaval shunts in man(2,3). Several reports(3-9) have described gross disturbances in ammonia metabolism in cases of liver disease with central nervous system symptoms. The existence of spontaneous or surgically constructed portacaval shunts provides a mechanism whereby ammonia absorbed from the gastrointestinal tract may accumulate to abnormal levels in the peripheral blood, but the manner in which this abnormality might interfere with the normal metabolic functions of the central nervous system is not clear. Despite this apparent connection between elevated ammonia levels in the peripheral blood and disturbances in brain function, there is little evidence available on ammonia levels in the cerebrospinal fluid. Riebling(10) reported that elevations in ammonia level in cerebrospinal fluid occurred in patients with epilepsy who were having convulsive seizures. Harris(11) reinvestigated this problem and was unable to confirm these observations. He pointed out that the method of measuring ammonia employed by Riebling did not take into consideration the release of ammonia from glutamine, known to be present in cerebrospinal fluid. Using Conway's micro-diffusion technic of measuring ammonia(12), he concluded that no free ammonia exists in normal cerebrospinal fluid nor is there an elevation of ammonia in the presence of convulsive seizures. Richter and associates(13) reached similar conclusions. The only record of ammonia content in the cerebrospinal fluid in hepatic disease is made by Fuld(14) who failed to detect any free ammonia in the one patient he observed.

Methods. Patients undergoing surgery which required a spinal anesthesia and who had no demonstrable liver disease were utilized as controls. Simultaneous measurements were made of ammonia levels in peripheral blood and in cerebrospinal fluid. To study ammonia metabolism in patients with hepatic disease, 22 simultaneous measurements of ammonia levels in blood and spinal fluid were made on 14 patients undergoing surgery for portal hypertension or who were under observation and treatment for known liver disease. Levels of ammonia in both blood and cerebrospinal fluid were determined by a previously reported modification(2) of the microdiffusion technic described by Conway (12). In this method a 10-minute diffusion time is employed that automatically eliminates any appreciable error which might be caused by liberation of ammonia from glutamine. Richter and his co-workers(13) have shown that the rate of liberation of ammonia by alkaline hydrolysis of glutamine is .001 mg ammonia per mg glutamine per hour at 20°C. Similar observations have been made in this laboratory and it is therefore felt that the existence of glutamine in cerebrospinal

TABLE I. Ammonia Concentration in Normal Human Blood and Normal Cerebrospinal Fluid.

Normal human blood	
No. of subjects examined	20
Range	44-71 γ %
Mean	55.6 γ %
Normal cerebrospinal fluid	
No. of subjects examined	7
Range	0-14.7 γ %
Mean	6.4 γ %

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* These studies supported by grant from the National Institutes of Health, U. S. Public Health Service.

† Bilton Pollard Fellow, University College Hospital, London. Present address: Royal Infirmary, Manchester, England.

TABLE II. Simultaneous Measurements of Ammonia Levels in Blood and Cerebrospinal Fluid in Patients without Evidence of Liver Disease or Portal Hypertension.

Patient	Disease and/or operation	Date	μg NH_3-N/100 cc Blood	μg NH_3-N/100 cc CSF
C.T.	Orchidectomy and transurethral resection for cancer of prostate	6/24/53	51	9
A.P.	Transurethral resection for benign prostatic hypertrophy	26	65	4.8
A.S.	Ureterolithotomy	30	54	14.7
A.V.	Appendectomy	7/ 8	59.3	2.7
G.T.	Suprapubic prostatectomy for benign prostatic hypertrophy	8/12	60	7.8
R.C.	Ureterolithotomy	20	51.5	0

TABLE III. Simultaneous Measurements of Ammonia Levels in Blood and Cerebrospinal Fluid in Patients with Portal Hypertension and/or Liver Disease.

Patient	Disease and/or operation	Date	μg NH_3-N/100 cc Blood	μg NH_3-N/100 cc CSF
E.E.	During portacaval shunt for portal hypertension	1/ 8/53	100	26
		8	102	35
E.S.	Terminal phase of alcoholic cirrhosis. Stuporous	16	192	153
M.G.	Alcoholic cirrhosis. Progressive disorientation and stupor	6/26	132	16
		27	173	52
R.N.	Alcoholic cirrhosis in coma. Recovering	26	326	213
		28	117	92
T.T.	During splenorenal shunt for cirrhosis and portal hypertension	2/ 1	99	36
		1	191	54
T.G.	*Idem*	3/13	103	89
E.G.	"	18	130	62
		18	109	71
L.W.	"	24	95	52
		24	66	55
S.B.	Cirrhosis of liver on high protein diet. Confused and stuporous	31	267	162
		4/ 7	327	209
L.C.	Cirrhosis of liver. Recovering from coma	7	170	116
L.L.	During portacaval shunt for cirrhosis of liver	5/ 4	152	105
		4	115	90
F.F.	4 yr after portacaval shunt. Chronic confusion and apathy	5	240	99
M.P.	During splenorenal shunt for portal hypertension and cirrhosis	6/ 1	136	33
A.Z.	*Idem*	23	135	58

fluid does not introduce an appreciable error in the measurement of ammonia levels if this technic is employed.

The *results* of these observations are shown in Tables I, II, and III, and the correlation between levels of ammonia in peripheral blood and in cerebrospinal fluid is shown diagrammatically in Fig. 1.

In patients used as controls and whose peripheral blood ammonia levels were within the normal range for this method, no appreciable amounts of ammonia could be detected in the cerebrospinal fluid (Table II). It is felt that the very small amounts (up to 14.7 μg %) represent an inevitable minor contamination with tissue fluids during manipulation of introducing a needle into the spinal canal. Therefore these observations are in complete agreement with those of Harris who found no appreciable amounts of ammonia in cerebrospinal fluid.

In patients with known disease of the liver or portal circulation, however, a different situation existed. All these patients except

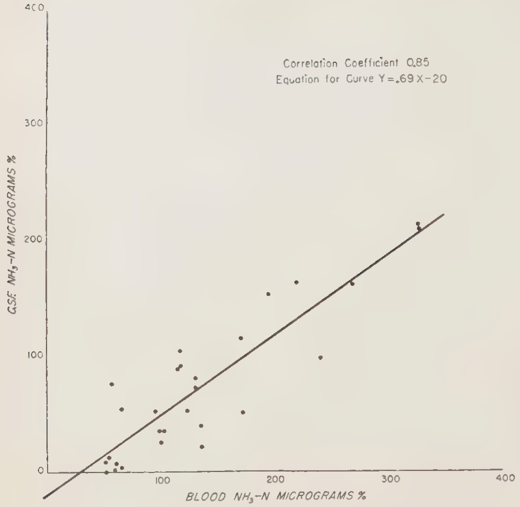

FIG. 1. Correlation between simultaneous measurements of ammonia in blood and cerebrospinal fluid in all patients (with and without liver disease) studied.

one had abnormally elevated ammonia levels in the peripheral blood, and measurements of ammonia levels in cerebrospinal fluid showed that there was a corresponding elevation although the levels were consistently lower than those found in peripheral blood (Table III).

In Fig. 1 the correlation between ammonia levels in peripheral blood and in cerebrospinal fluid is charted diagrammatically. The line calculated to show the relationship between these levels demonstrates this excellent correlation. This line however, does not pass through the zero point but crosses the abscissa, which represents the peripheral blood level, at 30 μg %. The significance of this observation will be discussed later.

Discussion. In confirmation of previous reports no appreciable amount of ammonia was found in cerebrospinal fluid of normal individuals. In patients with disease of the liver and portal circulation, however, distinct elevations of ammonia levels in the cerebrospinal fluid occur, and these levels bear a direct relationship to concomitant elevations of ammonia as measured in peripheral blood. In no case however, was the ammonia level in spinal fluid as high as in peripheral blood.

These observations suggest that in normal individuals there is a substance in peripheral blood which can liberate ammonia under conditions of this hydrolysis and that this substance does not exist in cerebrospinal fluid. In all probability this substance is adenosine as has been suggested by Conway(15). Under conditions in which there is a disorder in ammonia metabolism however, and abnormal levels are found in the peripheral blood, a corresponding though lesser elevation occurs in cerebrospinal fluid. Presumably therefore, regardless of the chemical form in which this abnormal ammonia is transported in the circulation, it is consistently diffusible into the cerebrospinal fluid. From these data it is not possible to say whether or not the abnormal elevations in ammonia are the result of or the cause of the disturbances in brain physiology which obviously exist in the presence of severe liver disease. Nonetheless, correlating these observations with the data previously reported from this and other laboratories, it would appear that in the presence of disease of the liver or portal circulation ammonia metabolism is disturbed in such a way as to permit abnormal elevations in peripheral blood. These elevations are reflected in abnormal concentrations of ammonia which exist in cerebrospinal fluid under these same conditions, and in all probability some of the neurological symptoms attendant upon severe liver disease or upon the existence of spontaneous or surgically constructed Eck fistulas are directly due to the toxic effect of ammonia on the central nervous system. It is relevant to point out that all 5 cases in Table III in which the spinal fluid ammonia level was greater than 105 μg % had significant disturbances of the central nervous system.

Conclusions. 1. No appreciable ammonia exists in the cerebrospinal fluid of normal individuals. 2. In disease of the liver or portal circulation abnormal elevations in the level of ammonia in peripheral blood may occur, and these are accompanied by corresponding but lesser elevations in levels of ammonia in the cerebrospinal fluid. 3. The significance of these observations is discussed.

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Received February 9, 1955. P.S.E.B.M., 1955, v88.

Assay of Breath Carbon-14 Dioxide of Humans Using Ionization Chambers.* (21595)

ELTON M. BAKER, B. M. TOLBERT, AND M. MARCUS.
From University of California Radiation Laboratory, Berkeley.

Since the use of carbon-14 as a tracer element in humans has become accepted and important(1-4), more adequate methods for low level radioactive measurements have been developed(5-8). This has been necessary because in experiments in man, high dilution of injected activity is common and radioactive doses are limited by health hazards. In this paper, equipment is described for the separation by a physical method of carbon dioxide from the expired breath of patients given C^{14}-labeled compounds and the radioassay of this CO_2 by a series of large size ionization chambers. This method depends on the analysis of large samples which permits in spite of low specific activity C^{14}, reliable assay of radioactive samples.

A normal human breathes some 5-7 liters of air per minute, which contains some 100 to 200 cc of carbon dioxide at NTP. Almost all of this carbon dioxide comes from his body metabolism in a well ventilated room. It is a slow process to absorb in alkali the carbon dioxide from this air, precipitate the CO_2 as barium carbonate, which is finally converted into pure CO_2 to be introduced into large size ionization chambers (500 cc and larger). Furthermore, such a procedure usually introduces a large radioactive contamination, largely due to radon, which is in equilibrium with the trace amounts of radium that are present as contaminants even in carefully purified barium or calcium compounds.

Experimental. Equipment for the direct separation of the CO_2 from the air by physical means on a vacuum line has been devised and is shown in Fig. 1. In this apparatus a 2-way valve system admits fresh air to the patient's face mask or B.M.R. mouthpiece and passes this air out through a large (10 mm bore) stopcock, a short ice water cooled condenser, and into the large balloon.† Air is withdrawn from this system through trap A, which is cooled in liquid air or nitrogen and exhausted to a house vacuum. The construction of this trap is rather important, as it must be able to condense all the CO_2 from air which should conveniently flow through at about 5 liters/min. The quantitative condensation of the CO_2 is accomplished by means of the spiral trap coupled with a sintered glass disk filter. Much of the condensible material in the air is cooled to a solid particle state suspended in the air. At

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* The work described in this paper was sponsored by the U. S. Atomic Energy Commission.

† Weather Bureau observation balloon, about 2 feet diameter when filled loosely.

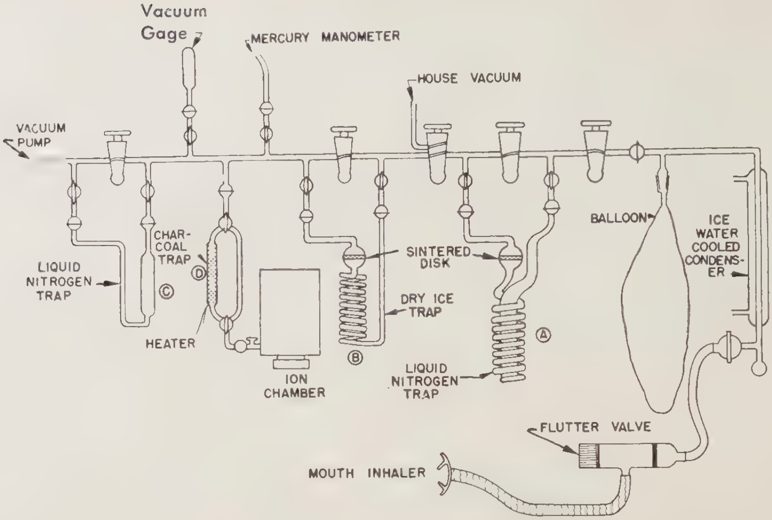

FIG. 1. Vacuum system for direct separation of CO_2 from exhaled air.

high flow rates of gas these are normally blown through a trap, but the addition of the sintered disk filters out these particles, and an excellent recovery of the CO_2 is achieved(9). This step separates nitrogen and oxygen from CO_2, water, and other gases condensible in a liquid nitrogen cooled trap. After a desired amount of air has been processed, trap A is evacuated and the contents allowed to warm and pass through trap B, which is cooled in an isopropyl alcohol-Dry Ice mixture, and finally condenses in trap C, which is cooled with liquid nitrogen. Trap B is constructed similarly to trap A, but may be made of smaller size glass tubing. This step removes water from the carbon dioxide. The CO_2 is then allowed to volatilize into the closed-off evacuated system, and in so doing passes through a 1 x 10 cm section of activated charcoal, D,[‡] until the ionization chamber is filled to 76 cm Hg pressure. The absorption of CO_2 on this charcoal trap is not large and its important function is to remove radon, a short half-life alpha radioactive element. *Radon* has melting and boiling points very close to the sublimation point of CO_2 and is not removed by any of the previous steps. The amount of radon in the air varies a great deal and depends on the nature of the surroundings. Thus, concrete buildings in the West have been noted for large radon concentrations due to the uranium content of many of the western sands. Unless the radon is carefully removed from the CO_2 in the experiments described in this paper, no correction for α emission can be made and the sensitivity of the method is greatly decreased. The radon in the carbon dioxide, however, may be easily absorbed on clean degassed charcoal at room temperatures. Furthermore, it is not necessary to use new charcoal for each sample, since radon may be desorbed by heating the charcoal to 200°C in an evacuated system (10 μ pressure). This heating is easily accomplished by wrapping the charcoal section with a nichrome wire in asbestos which can be electrically heated.

The ionization chamber measurements discussed in this paper were made with a vibrating reed electrometer[§] and continuous recorder[‖] using the rate of drift method(10). An automatic grounding mechanism returns the center electrode to ground potential whenever it reaches the limit of the chart scale, so that continuous traces for several hours at a

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[‡] The activated charcoal was taken from a gas mask chemical cartridge, Cat. No. DR-44135, Mine Safety Appliances Co., Pittsburgh, Pa.

[§] Vibrating reed electrometer, Applied Physics Corp., Pasadena, Calif.

[‖] Brown potentiometer recorder, 0-25 mv., Minneapolis Honeywell Regulator Co., Philadelphia, Pa.

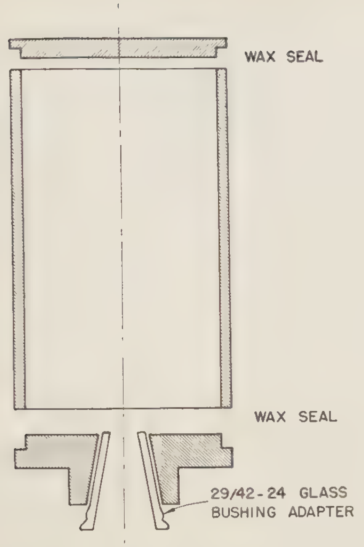

FIG. 2. Cylindrical ionization chamber with a 29/42-24/40 short glass bushing adapter.

time can be made and analyzed. In the analysis the drift with and without α's is measured for 10-minute intervals for at least 2 hours. By this method any instrument variations such as insulator hysteresis currents can be detected. Only after constant drift rates are reached is the data used.

A series of chambers were constructed from 450 to 1500 cc in volume and compared with a 100 cc chamber in common usage(5,7). The sizes and characteristics of the chambers are listed in Table I and the general design shown in Fig. 2. The collecting electrode insulators were purchased from the Applied Physics Corporation¶ and were equipped with a sapphire insulator.

Discussion. The background current of an ionization chamber is due to insulator drift current plus ionization current derived from cosmic rays, radiation emitted from chamber walls and activity in the chamber gas. When an ionization chamber current is analyzed with a sensitive electrometer and continuous recorder, the α contribution derived from the chamber walls and gas may be subtracted if such events are not numerous. The effects of other radiations can not be so easily eliminated.

The efficiency of the production of ion pairs in an ionization chamber at constant pressure for soft β radioactivity will increase with size of the chamber, rapidly if the dimensions of the chamber are small compared to the range of the particle, and less rapidly when the beta range is small compared to chamber size.

As the size of the ionization chamber is increased, therefore, the sensitivity of the radioactive assay will be increased due to the following factors: 1) increased precision of measurements of electrical potentials for higher drift rates, achieved mostly through reduction in importance of short term instrument variation, such as tube noise and insulator hysteresis; 2) more efficient absorption of β particles in the gas, and therefore more ion pair production per beta particle; 3) relative decrease in background from chamber wall material as compared to activity from the sample. Increasing the size of the sample does not influence the ratio of sample activity to cosmic ray background.

For a given set of physical conditions there must be, therefore, a certain size of ionization chamber, beyond which further volume increase will give only slight advantages. The major source of error at low drift rates in ionization chambers is instrument variation, probably mostly due to insulator hysteresis. This effect may be largely due to deformation of the insulator by pressure changes(11). When this variation in drift rate, ± 0.05 mv/min., is equal to the precision of the electrical instruments, which is about ± 1%; further size increase gives no improved sensitivity. The limiting sensitivity for this work occurs at about 4 mv/min. which is the drift rate for the 1000 cc chamber.

In carbon dioxide at one atmosphere pressure the maximum range of the C^{14}-β particle is 15.5 cm and the average range is 5 cm ($E_{max} = 0.154$ mev., or 27.9 mg/cm^2, Eav $= 0.050$ mev. Density of CO_2 is about 1.8 mg/cm^2 at 25°C). The dimensions of the 1000 cc ionization chamber are of the same order of magnitude as the average range of the C^{14}-β particle, in agreement with the data in Table I. The sensitivity of this size chamber (about 0.001 dis/min./mg $BaCO_3$) makes it quite possible to carry out extensive

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¶ Applied Physics Corp., Pasadena, Calif.

TABLE I. Chamber Size *vs.* Collection Voltage and Sensitivity.*

Chamber size			Beginning of plateau voltage†	Voltage used	Sensitivity in dis./min./mg $BaCO_3$	Equiv. value of background in dis./min./mg $BaCO_3$	Bg in mv/min. drift rate
Diam.	Length	Vol, cc					
.70″	2.75	100	22	90	.01	.07	.50
3.25	3.0 ‡	450	135	270	.004	.04	1.80
3.25	3.5	500	135	270	.004	.05	2.20
4.75	3.5	1000	30	60	.001	.04	4.10
3.75	5.5	1000	30	60	.001	.04	4.20
5.25	4.25	1500	30	60	.002	.04	6.40
42.5	6.5	1500	90	180	.002	.04	6.30

* All values are with α background subtracted.

† Distance between end of the probe and tip of the chamber were not kept constant.

‡ Made with a rounded top of 1.5/8″ radius.

experiments in humans with as little as one microcurie of carbon-14.

Summary. An apparatus for the physical separation and purification of CO_2 from human breath is described. These CO_2 samples have been measured in a series of ionization chambers varying from 100 to 1500 cc. The optimum sample size was found to be 1000 cc for these unshielded-atmospheric pressure ionization chambers. With such equipment it is possible to carry out experiments in humans with as little as one microcurie of carbon-14.

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Received February 9, 1955. P.S.E.B.M., 1955, v88.

Metabolism of Glycine-2-C^{14} in Man: V. Further Considerations of Pulmonary Excretion of $C^{14}O_2$.* (21596)

N. I. BERLIN AND B. M. TOLBERT.

From Section on Research Medicine, Donner Laboratory and Radiation Laboratory, University of California, Berkeley.

In a previous communication it was reported that the rate of excretion of C^{14} via the lungs, as $C^{14}O_2$, following the administration of glycine-2-C^{14}, may be conveniently described in terms of 3 rate processes having average time of 2.9 hours, 31 hours and 240 hours, respectively(1). It was postulated that another component of C^{14} pulmonary excretion was present, having a half time greater than 90 days. Autopsy data on one patient at 526 days after administration indicated that there was a measurable amount (approximately 1% to 2% of the administered amount of C^{14}) present at that time(2). It was also reported that the urinary C^{14} excre-

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* These studies supported by grant from the U. S. Public Health Service and the U. S. Atomic Energy Commission.

TABLE I. Percent of Administered Dose Excreted and Half Time for Each of 5 Rate Processes.*

		I		II		III		IV	
Patient	(R) (min.)	T½ (days)	%	T½ (days)	%	T½ (days)	%	T½ (days)	%
W.G.	12	.09	14.5	1.2	17.2	7.5	29.8	114	24.6
E.H.	19	.11	24.3	1.1	13.9	9.8	27.9	134	20.1
J.M.	16	.10	16.2	.8	16.2	3.4	24.8	38	24.8
W.G.	6	.12	15.5	1.5	17.7	4.6	19.8	38	32.6
E.F.	28	.19	33.5	.9	7.7	4.2	22.8	54	30.6
F.W.	10	.10	21.0	.3	12.6	7.0	28.6	51	22.0
Avg		.12	20.8	.97	14.2	6.1	25.6	71.5	25.7

Pulmonary excretion and half time values for metabolism of C-14 administered as glycine-2-C-14 in man.*

* The slope of component (R) is positive, while the other 4 components are, of course, negative.

tion amounts to approximately 12% of the dose, and could be conveniently described in terms of 3 rate components having average half times of one-quarter, 2½ and 53 days, respectively(3). At that time, it was thought that approximately 98% of the administered C^{14} could be accounted for in terms of the then known pulmonary and urinary excretion rates. With improvements in the method for measuring the specific activity of the exhaled carbon dioxide, it became possible to study the long term excretion of C^{14} in the breath. The present study was designed to determine if there was a component of excretion in the breath with a half time longer than 10 days, and to measure its magnitude.

Methods. Six patients given methylene C^{14} labeled glycine (100 μc in 8-12 mg glycine) for the determination of the life span of the red cell were studied. For the first 18 to 20 samples the CO_2 was absorbed in sodium hydroxide solution and then precipitated as barium carbonate, as reported previously(1). Later samples were collected by a method designed to remove the carbon dioxide directly from the exhaled breath and then to fill the ionization chamber with this carbon dioxide(4). This method permits the collection of a breath sample over a 10 minute period and subsequent use of a 500 cc ionization chamber, as compared to a 100 cc ionization chamber. This larger ionization chamber permits the lowering of the limit of resolution for the measurement of $C^{14}O_2$ by approximately a factor of 10. The 6 patients were followed up to 330 days after the administration of 100 μc of glycine-2 C^{14}.

Results. Results are presented in Fig. 1 and 2, and Table I. Fig. 1 shows the specific activity of the expired carbon dioxide as a function of time in patient #1 for the slowest 3 components. This study shows that there is indeed a component of pulmonary excretion with a half time greater than 10 days. This longer component has a half time of from 38 to 134 days and averages 71.5 days. Integration of the area under the curves for the specific activity shows that in these patients an average of 25.7% (with a range of 20% to 33%) of the C^{14} that is excreted through the breath is excreted in this slowest (fourth) component.

In the first communication, it was stated that the maximum specific activity of the expired breath occurred within the first hour following injection. In these 6 patients, a sufficient number of samples were taken to

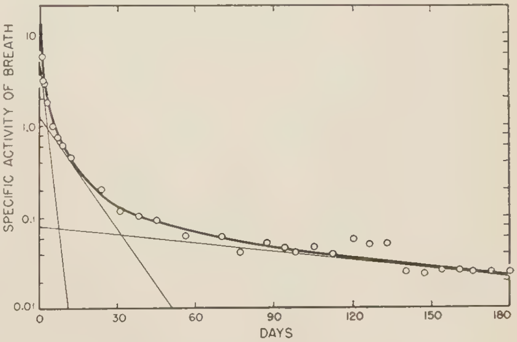

FIG. 1. Specific activity of the breath in patient #1 in dis./min./mg/$BaCO_3$. Decreasing component 1 is not shown.

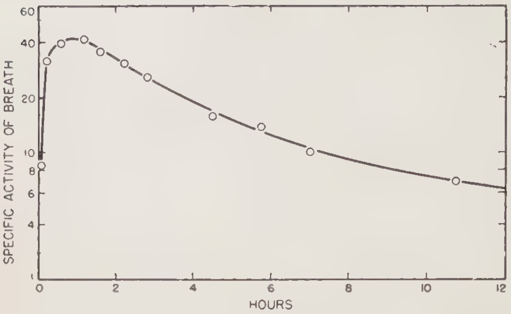

FIG. 2. Specific activity of the breath in patient 1 for the first 12 hours in dis./min./mg/$BaCO_3$. Increasing component and decreasing component 1 are shown.

measure the rate at which this maximum occurred. Figure 2 shows this portion of the curve of patient #1. For analysis it may be assumed that for this brief period of time, one to 2 hours after administration, that the curve represents one which can be described by the following equation:

$$N_t = N_f \ (1\text{-}e^{kt})$$

where

N_t = specific activity at time t.
N_f = maximum specific activity.
k = rate constant.

The half time for the rising portion of the curve has an average value of 15 minutes.

Discussion. Since in previous communications(1-3) it was reported that 98% of the administered C^{14} could be accounted for by the first 3 rate processes in the pulmonary excretion and by the complete urinary excretion, it becomes evident with the finding of the fourth pulmonary component of C^{14} excretion that the amount that can be accounted for becomes approximately 15% to 20% greater than the amount administered. A careful review of the methods of the calculation of the amount excreted shows there is one significant fact that may be consistently overestimated. For the integration of the area under the curve in Fig. 1 and 2 to determine the cumulative C^{14} pulmonary excretion, it is necessary to know the total amount of carbon excreted per minute. This has been determined in all the patients reported here and previously by collecting the breath through a device shown in the first paper and measuring the amount of barium carbonate produced in a measured period of time. It was thought by taking a large number of samples (20) over a period of 10 days after administration that a reasonably average figure would be obtained. Although the data shows some variation from sample to sample in given individuals, what is probably more important is most of these samples were taken during the waking hours, and in computing the average we have not included the sleeping hours when the rate of production of CO_2 may be decreased in these patients. A second factor not previously known to be as significant as it now appears, is the influence of extraneous stimuli on the rate of CO_2 production. Siri(5) in this laboratory, using a modification of the thermal conductivity gas analysis method of Berg and Cook(6) has shown that mild external auditory or visual stimuli, such as the closing of a door, are capable of producing a significant increase in the rate of CO_2 production in resting individuals. It has been further noted that some patients, despite careful instructions to breathe naturally, will attempt to do a good job by filling the balloon.

In graphically integrating the values obtained in the first 4 patients(1), the findings of the slowest component, which has an average half time of 10 days, should be compared with the average half time, 6.1 days, determined for that component in the presently reported 6 patients. The graphic calculation of this third component, as originally reported, was undoubtedly influenced by the presence of the slowest component which was too small to measure directly. The net result of having this slowest component (fourth) included in the calculation of the half time for the third component is to decrease the slope and thus give a longer half life.

There are comparatively few studies of the variation of the rate of CO_2 production during the day(7-9). However, it has been clearly demonstrated in the few individuals studied that the CO_2 production rate at night is considerably lower than in the day(8,9). There is one study in a patient with leukemia in which the CO_2 production during the night time was considerably greater than has been reported for normals. However, this data is quite old, 1869, and only one individual was studied.

A third factor is the possibility of a systematic error in the radioactivity determination, especially at low activity levels. Analysis of the methods used does not show any consistent error trend, but integration of small values for long periods of time may lead to appreciable cumulative errors. Previous studies with animals have shown that a quantitative accounting of the administered dose, both excretion and amount present in the tissues, will often vary from 95% to 110%. Thus, in the present instance, we have not exceeded the limits of excretion which have been found in animal studies.

These factors: 1) Taking the samples during the day time when the excretion of CO_2 may be higher than in the night time, and 2) the psychological factor of breathing into a balloon and the effect of extraneous stimuli all tend to increase the rate of production of CO_2, and thus, probably accounts in large measure for the over-estimation of the amount excreted.

While this finding of a fourth breath component will influence the calculation of the average radiation dose delivered to tissue shown previously(3), it will merely tend to shift the curves somewhat, so that a longer period of time will be required to reach the level of background radiation (Fig. 3). It does not in any way negate the general conclusion that after a period of time (approximately 200 days) the average C^{14} specific activity of all the tissues will reach a value equivalent to that of natural background radiation. This demonstrates that this long-lived isotope, may be safely used in clinical investigation, provided the compound is either excreted or metabolized.

It is now believed that we have presented an overall picture of the pulmonary C^{14} excretion, and that it may be described in terms of one increasing component and four decreasing components.

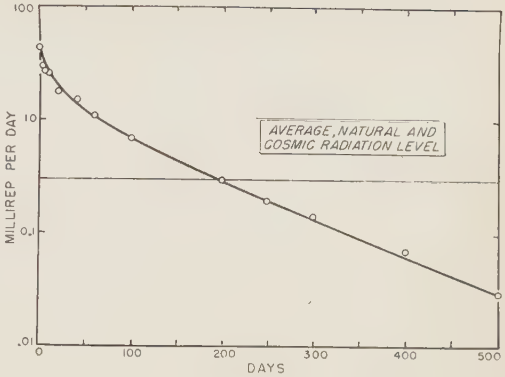

FIG. 3. Avg tissue radiation following administration of 100 mc of C-14 as glycine-2-C-14.

Conclusion. The fourth component of pulmonary excretion after the intravenous injection of glycine-2 C^{14} has been determined and found to have an average half time of 71.5 days. Recalculation of the half time and associated C^{14} excretion shows that decreasing component I has an average half time of 0.12 day and that 20.8% of the C^{14} is excreted with this rate process. For the others the half times and percentages are 0.97 day, 14.2%; 6.1 days, 25.6%, and 71.5 days, 25.7%.

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Received February 9, 1955. P.S.E.B.M., 1955, v88.

Maintenance of Respiratory Function with Isolated Lung Lobes During Cardiac Inflow Occlusion.* (21597)

Gilbert S. Campbell,† Norman W. Crisp, Jr., and E. B. Brown, Jr.
From Departments of Surgery and Physiology, University of Minnesota Medical School, Minneapolis.

There have been many different approaches towards the goal of open cardiac surgery in humans, and significant contributions have been made by numerous investigators. Sixteen years ago Gibbon(1) reported prolonged healthy survival of dogs in which all the blood flow through the heart and lungs was completely interrupted by clamping the pulmonary artery for as long as 20 minutes, while cardiorespiratory functions were maintained by an extracorporeal circuit. Mechanical extracorporeal pump-oxygenators have received widespread experimental study(2-4) but as yet clinical success with such systems is relatively rare. Others(5-7) have attempted total cardiac by-pass by means of mechanical pumps and biologic oxygenators (autologous and homologous lungs). Pulmonary edema in biologic oxygenators as well as post-operative hemorrhagic tendencies in experimental animals carried on either artificial or biologic oxygenators have been troublesome. Andreasen and Watson(8) established the azygos flow concept, *i.e.* ability of dogs to withstand 30 minutes of complete vena caval occlusion if the flow through the azygos vein into the heart was maintained, and these same workers described the controlled cross-circulation method(9). This work was confirmed and clinically applied by Warden, Cohen, Read and Lillehei(10).

The current investigation was undertaken in an attempt to develop a technic of cardiac by-pass which would embody the principles of Andreasen and Watson but circumvent the use of a donor.

Methods. Unaged stock mongrel dogs were intravenously anesthetized with 2.5% sodium pentobarbital (25-30 mg/kg) and given intravenous atropine grains 1/100 preoperatively. Intermittent positive pressure ventilation was administered through a cuffed endotracheal tube and thoracotomy was made through the right fifth interspace. The vena cavae and azygos vein were identified and large ligatures looped loosely around these vessels (Fig. 1). Either the right cardiac or the right diaphragmatic lung lobe was removed and placed in

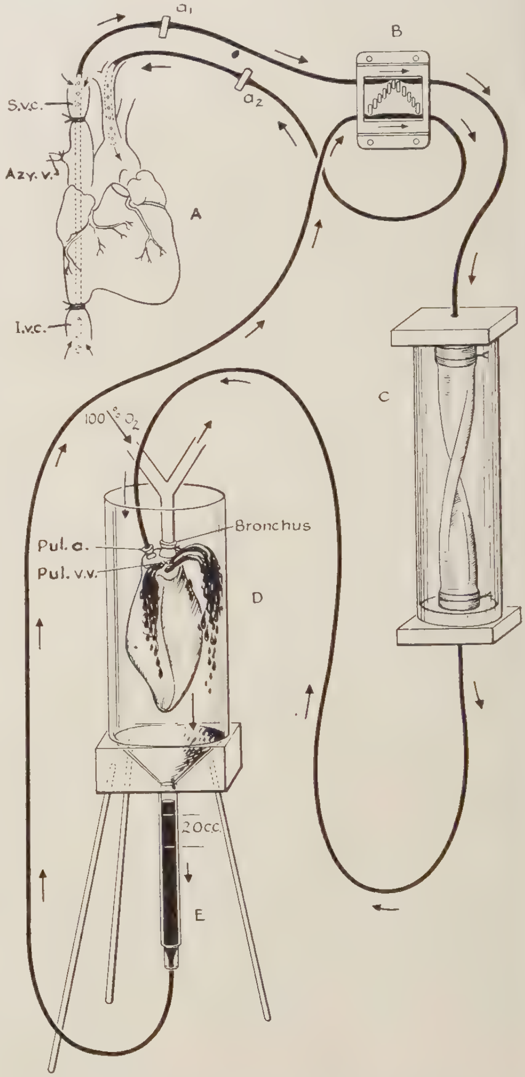

FIG. 1. Venous blood aspirated from vena cavae (A) through pump (B) to depulsator (C) to pulm. a. of lung in chamber (D) via open pulm. vv. to flow-meter and reservoir (E), and thence to pump (B) and to aorta (A).

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* This investigation supported in part by grant from the U. S. Public Health Service and Graduate School, University of Minnesota.

† Markle Scholar in Medical Sciences.

TABLE I. Total Cardiac By-Pass Utilizing Autologous Lobe Oxygenator, in 10 Dogs. Immediate survival in all.

Wt (kg)	By-pass flow (cc/kg)	Early deaths (<4 wk)	Long term survivors
18.6	17		Yes
14.1	30	3 hr*	
15.9	38	24 " D†	
17.7	34	5 days D	
18.2	33	*Idem*	
16.8	36	"	
20.9	28		Yes
17.7	34		"
15.0	40		"
14.1	27		"

* Air embolism. † D = Distemper.

the methyl methacrylate chamber. The extracorporeal circuit was primed with sterile normal saline in the arterial side and with blood in the venous side (*i.e.* the side leading to the isolated lung lobe which had a cannula in its pulmonary artery). The capacity of the extra-corporeal system was 175-200 cc depending on length of plastic tubing used. A large cannula was inserted into the bronchus of the isolated lobe and the lobe subsequently ventilated with 100% oxygen at a rate of 12-18/minute with intermittent positive pressure of 14-16 cm of water. The dogs were given heparin intravenously (1 mg/kg body wt). Plastic cannulae (#12-14 French) were inserted into the right jugular vein and right carotid artery of the experimental animals and positioned as in Fig. 1. After clamping the azygos vein, the pump was started and the venous pick-up from the animals' vena cavae was equated against the arterial return to the animal's aorta by tightening either clamps a_1 or a_2 (Fig. 1). Concomitant with starting the pump, the isolated lung lobe was ventilated and the dog disconnected from the respirator. Within a few minutes the tapes on the vena cavae were tightened thereby initiating total cardiac by-pass. Total cardiac by-pass was maintained for 30-35 minutes and blood samples drawn just prior to termination of total by-pass period. Arterial blood samples were drawn from the left femoral artery cannula which was otherwise connected to a Statham Strain gauge for blood pressure recordings, and venous samples were drawn simultaneously from the venous limb of the extracorporeal system. *Perfusion pressure* in the pulmonary artery of isolated lobe was measured with a Statham Strain gauge, and in the latter part of study a depulsator was employed on the pulmonary artery limb of the circuit to protect the lobe from high pump ejection pressure transmitted through the rigid extracorporeal plastic tubing. After release of the vena cavae tapes, the pump was kept in motion for 1-2 minutes and the residual blood in the arterial limb of extracorporeal circuit was thus returned to the dog. Protamine sulfate (1 mg/kg) was administered intravenously in 100 cc normal saline over a 5-10 minute period after release of the by-pass, and in most cases blood transfusions were not given. The neck cannulae were removed after the by-pass and the jugular and carotid vessels ligated. The dog's rectal temperature dropped 2 to 3°C during the by-pass, and no attempt was made to offset this temperature drop. The chest wound was closed and residual air and/or fluid was removed via a chest catheter. These experiments were carried out under clean but not strictly aseptic conditions. *Blood pH* was measured with a glass electrode under anaerobic conditions at 37°C(11) and total blood CO_2 content determinations were made with the Van Slyke manometric apparatus. Knowing the pH and the total CO_2 content, CO_2 tension, carbonic acid and bicarbonate concentrations were calculated utilizing the Henderson-Hasselbalch equation. Platelet counts, clotting times (Lee-White), prothrombin times (Simplastin) and hemoglobins were determined in some experiments. Electrocardiograms were taken simultaneously with femoral artery pressure recording of the dog and the pulmonary artery perfusion pressure of the isolated lung lobe. Blood flows during total cardiac by-pass were measured after

TABLE II. Total Cardiac By-Pass Utilizing Autologous Lobe Oxygenator.

	Control	End of by-pass		Recovery
	arterial	Arterial	Venous	arterial
pH	7.32	7.25	7.16	7.33
CO_2 (mM/l)	22.1	15.6	21.4	19.4
pCO_2 (mm Hg)	43	37	59	38
HCO_3^- (mM/l)	20.8	14.5	19.9	18.5
O_2 (vol %)	20.7	20.8	7.1	17.9

equating venous aspiration from the dog with arterial return to the dog. Then by clamping the venous pick-up side from the dog, the blood level in the vertical tube below the lung chamber would fall, and the rate of fall was timed with a stop watch. Several flow measurements were made during the 30-35 minute by-pass periods. No cardiotomies were done.

Results. Ten dogs were subjected to 30 minutes of total cardiac by-pass employing a mechanical pump and an isolated autologous lung lobe oxygenator (Table I). The average flow was 32 cc/kg/min. and with this decreased flow the dogs' systolic blood pressure dropped from a control of 155 mm Hg to 75 mm Hg. Upon release of the inflow occlusion to the right heart the systolic blood pressure rose to 140 mm Hg within 10 minutes. Arterial blood pH fell 0.07 unit during the 30 plus minutes of extra-corporeal cardiorespiratory maintenance. Table II shows the degree of metabolic acidosis and the increased arterio-venous oxygen differences resulting from the decreased flows. Pulmonary edema was absent or relatively slight in the 10 autologous lobes. The average increase in lung lobe weight was 40 g and the adequacy of its gaseous exchange ability at the *end* of the by-pass is supported by the data in Table II.

Homologous isolated lung lobes were used as oxygenator in 10 dogs (Table III) and these homologous lobes functioned as well as the autologous lobes as shown by Table IV. Death from cerebral edema was probably due to repeated occlusion of the venous pick-up limb from the dog for periods as long as 40 seconds. The imbalance in flows between the 2 limbs through the pumps necessitated such clamping to equate arterial return to the dog with venous aspiration from the dog. Arterial inflow was continued during these periods thereby perfusing the brain without venous drainage from the brain. This difficulty has subsequently been avoided by a separate pump for each circuit of the extracorporeal system.

TABLE III. Total Cardiac By-Pass Utilizing Homologous Lobe Oxygenator, in 10 Dogs. Immediate survival in all.

Wt (kg)	By-pass flow (cc/kg)	Early deaths (<4 wk)	Long term survivors
25.4	16		Yes
16.3	37		"
13.4	34	3 hr D†	
10.4	60		"
8.2	27		"
10.0	63		"
11.4	35	3 hr*	
12.7	39	24 " D	
15.4		48 " Asp. pneum.	
14.5	28	24 " D	

* Cerebral edema. † D = Distemper.

TABLE IV. Total Cardiac By-Pass Utilizing Homologous Lobe Oxygenator.

	Control arterial	End of by-pass Arterial	End of by-pass Venous	Recovery arterial
pH	7.33	7.26	7.18	7.22
CO_2 (mM/l)	24.2	14.7	19.5	16.0
pCO_2 (mm Hg)	48	31	57	39
HCO_3^- (mM/l)	22.8	13.9	17.8	14.8
O_2 (vol %)	18.3	19.5	6.9	16.9

In the first 4 homologous lobe experiments the depulsator was not used on the pulmonary artery limb of the extracorporeal circuit and the average weight gain in the lung lobes was 54 g. The depulsator was employed in the last 6 homologous lobe experiments and the average weight gain in this group was only 24 g.

The average extracorporeal flow in the homologous group was 38 cc/kg/minute and the systolic blood pressure dropped from a control level of 160 mm Hg to 75 mm Hg during the by-pass and returned to 120 mm Hg upon release of inflow occlusion. Mean pulmonary artery perfusion pressures of 20-40 mm Hg were noted throughout the 30 minute by-pass period.

Control clotting times of 4 minutes were prolonged to 27 minutes immediately after the intravenous administration of heparin (1 mg/kg), and the clotting time had fallen to an average of 11 minutes (autologous lobe group) and 8 minutes (homologous lobe group) by the end of the by-pass period. Intravenous protamine (1 mg/kg) lowered the clotting times to 6 minutes and none of the dogs in either group showed any hemorrhagic tendency. Prothrombin time increased from a control of 8 seconds to a post-operative level of 9 seconds and the blood platelet determinations for these periods were 187,000 and 100,000 respectively. Hemoglobin levels were essentially unchanged during the experiments, but perhaps the anticipated hemoconcentration was offset by fluid shift between extravascular and vascular compartments resulting from blood withdrawn into the extracorporeal system and for samples. No EKG changes were noted.

Discussion. Our results are in complete accord with the azygos flow concept—*i.e.* the ability of dogs to withstand extracorporeal cardiorespiratory maintenance with blood flows appreciably less than normal cardiac outputs.

Three major factors in our experiments which prevented or limited edema of the isolated lobe were (1) complete patency of the pulmonary veins—*i.e.* in order to prevent "back pressure" the veins were not cannulated, (2) depulsator between the pump and the pulmonary artery of the isolated lobe in order to protect the isolated lung from high pulmonary artery systolic pressures, (3) a sine wave type inflation of the isolated lobe at pressures of 14-16 cm of water. A direct result of these modifications was the ability of the isolated lobe to tolerate much higher flows than was possible in earlier experiments in which a "closed" system was used (with pulmonary veins cannulated)(12). Another advantage of the "open" system employed herein is the ease with which arterial return can be equated with venous aspiration from the dogs. This is a dynamic balance which requires constant monitoring due to changes in venous pick-up, alterations in resistance through the lobe, and variation in resistance at the site of arterial return to the animal. Furthermore, flows can be determined repeatedly by clamping the venous aspiration limb of the extracorporeal circuit and clocking the fall of the blood column in the vertical tube below the lobe housing.

Employing the 3 safeguards against pulmonary edema noted earlier; dog lungs were perfused for 30 minutes with human blood with flows of 300 cc/minute and remained in good condition. Further studies on stored homologous lungs are in progress.

Summary. 1. Dogs withstood and recovered from 30 minutes of total inflow occlusion to the right heart with extracorporeal cardiorespiratory maintenance at an average flow of 35 cc/kg/minute. 2. Biologic oxygenators (autologous and homologous lung lobes) and a pump (Sigma-motor) were used and the following precautions seemed essential (a) patency of the pulmonary veins—*i.e.* no cannulization whatever, (b) depulsator in pulmonary artery perfusion limb of the circuit, (c) care as to ventilatory mechanics of the isolated lobes. 3. The "open" system employed herein permitted rather exact balance between venous aspiration from bypassed dogs and arterial return to the dogs, and enabled flow measurements to be made.

Note added in proof. Since submission of this manuscript, human blood has been perfused through the isolated dog lung with flows up to 1400 cc/minute, and the clinical applicability of this system (pump-biological oxygenator) as a means of cardio-respiratory maintenance during cardiac by-pass has been tested by the successful closure of a traumatic interventricular septal defect in a 55 kg male. In this patient the heart was partially by-passed for 20 minutes and totally by-passed for 15 minutes.

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Received February 9, 1955. P.S.E.B.M., 1955, v88.

Biochemical Changes in Spleens of Rats after Localized X-Irradiation.* (21598)

DONALD F. PETERSEN, FRANK W. FITCH,† AND KENNETH P. DUBOIS.

From the U. S. Air Force Radiation Laboratory and the Departments of Pharmacology and Pathology, University of Chicago.

Previous studies in this laboratory(1) have shown that whole body x-irradiation produces increases in the adenosine triphosphatase and 5-nucleotidase activity of the hematopoietic tissues of rats of sufficient magnitude and reproducibility to be employed for quantitative estimation of radiation damage to these tissues. The applicability of phosphatase assays for evaluating the efficacy of chemical agents in protecting against radiation damage to hematopoietic tissues has been demonstrated recently by Petersen and DuBois (2). In the present investigation phosphatase measurements were employed to obtain further information concerning the influence of partial body shielding on radiation damage to hematopoietic tissues. The use of partial shielding also offered a method of determining whether radiation-induced biochemical changes in the spleen are the result of a direct action by x-ray on enzymes within the spleen. The results of this study demonstrated that the marked increases in the phosphatase activity(1), the decrease in citrate synthesis (3) and the decreased levels of desoxyribonucleic acid(4,5) which occur in the spleen after whole body irradiation are not observed when the exteriorized spleen is irradiated with the remainder of the body shielded. Histologic changes in the spleen were also much less pronounced after localized irradiation than following total body exposure.

Materials and methods. Adult male and female Sprague-Dawley rats were used for these studies. The animals were maintained in an air-conditioned laboratory (68-75° F) and were given Rockland rat pellets and water *ad libitum*. Shields designed to protect the body were constructed from 9-inch sections of lead pipe (0.5-inch wall thickness, 2-inch inside diameter) which were split longitudinally, hinged and slotted to permit exteriorization of the surgically mobilized spleen. X-ray penetration of the shields as measured with a Victoreen ionization chamber during an exposure period of 20 minutes was approximately 0.4% (3 r). Under pentobarbital anesthesia (35 mg/kg) the spleens were exteriorized through the left side of the animals. The exteriorized spleens were kept moist with physiological saline. The circulation of the spleen remained intact throughout the surgical manipulation and irradiation. There was no gross or microscopic evidence of infarction. X-irradiation was administered to the exposed areas at a rate of 38 to 40 r/min using a G.E. Maximar therapy unit. The radiation factors were: 250 KVP, 15 ma, 0.25 mm Cu and 1 mm Al added filtration, 75 cm target-specimen distance. Adenosine triphosphatase assays were performed by the method of DuBois and Potter(6) and 5-nucleotidase measurements were conducted according to the procedure of Cochran, Zerwic and DuBois(7) at various intervals after irradiation using cold water homogenates of spleen. Citric acid determinations were carried out on fluoroacetate-treated animals by the method of Natelson *et al.*(8) using the sequential blocking technic of Potter(9) except that the dose of fluoroacetate was reduced to 3 mg/kg to decrease the number of deaths due to fluoroacetate. The desoxyribonucleic acid (DNA) content of the spleen was measured by the method of Schmidt and Thannhauser (10) after removal of acid-soluble materials and phospholipids according to the method of Schneider(11). Material for histological examination was fixed in formalin, embedded in paraffin, sectioned at 6 μ and stained with azure A-eosin B(12).

Results. To ascertain whether localized irradiation of the spleen alters the phospha-

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* This study supported by funds provided under contract with the U. S. Air Force School of Aviation Medicine, Randolph Field, Texas.

† U. S. Public Health Service Postdoctorate Research Fellow in Pathology.

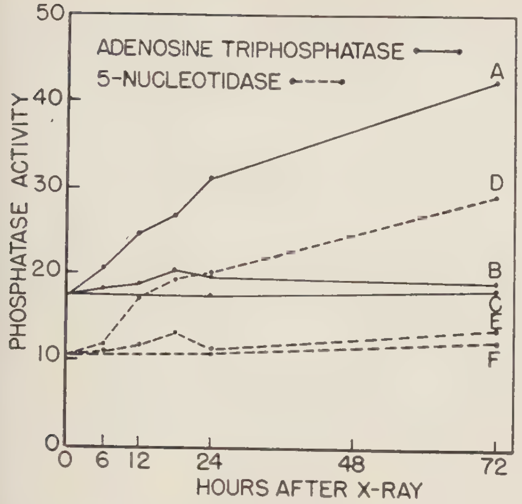

FIG. 1. Effects of partial shielding on adenosine triphosphatase and 5-nucleotidase activity of spleens of rats after 800 r of x-ray. Adenosine triphosphatase activity expressed as μg of P liberated from adenosine triphosphate/mg of spleen /15 min. and 5-nucleotidase activity is expressed as mg of phosphorus liberated from 5-adenylic acid/g of spleen/hr. A & D—whole body irradiation. B & E—spleen irradiated and body shielded. C & F—unirradiated.

tase activity of that organ a series of rats was given 800 r of x-ray to the exteriorized spleen with the remainder of the body shielded. Another series of animals which served as the irradiated controls received 800 r of total body irradiation administered with the spleens exteriorized. A third series of rats had the spleens exteriorized for a comparable length of time (*ca* 20 min) but received no exposure to x-ray. At frequent intervals from 6 to 72 hours after irradiation groups each containing four animals were sacrificed for adenosine triphosphatase and 5-nucleotidase measurements on the spleens. The results of these measurements are shown in Fig. 1. After whole body x-irradiation increases in the activity of both enzymes were detectable in the spleens as early as 6 hours after exposure and reached more than twice the normal level in 72 hours. However, direct irradiation of the spleen with the remainder of the body shielded resulted in no appreciable increase in the activity of either enzyme in the spleens throughout the observation period. Assays conducted on unirradiated animals in which the spleens had been exteriorized demonstrated that the surgical procedure did not materially alter the adenosine triphosphatase or 5-nucleotidase activity.

Following the demonstration of the absence of a direct effect by x-ray on the activity of phosphatases of the spleen similar experiments on citric acid synthesis *in vivo* were performed. For these measurements groups of 4 rats were given 800 r of whole body x-irradiation or the same dose of x-ray to the spleen with the remainder of the body shielded. At 1 day after irradiation groups of animals were treated with fluoroacetate and sacrificed 3 hours later for citric acid measurements on the spleens. The results shown in Table I indicate that localized irradiation of the exteriorized spleen does not affect its capacity to synthesize citric acid *in vivo* in contrast to the marked inhibition of citrate synthesis which occurs in the spleens of rats after whole body x-irradiation(3).

Additional experiments were conducted to obtain a comparison of the effects of whole body irradiation and localized irradiation on the DNA content of the spleens of rats. For these measurements groups each containing 4 animals were sacrificed at 24 and 72 hours after exposure of the exteriorized spleen to 800 r with the remainder of the body shielded. Whole body irradiated and unirradiated, spleen-exteriorized rats were included for comparison. The results of these measurements are shown in Table II. At 24 hours after irradiation of the spleen with the remainder of the body shielded only a small decrease in DNA concentration of the spleen was observed in contrast to a marked decrease to 44% of normal after whole body irradiation. Exteriorization of the spleen without subsequent irradiation had no effect on the DNA content of that organ.

TABLE I. Effect of Partial Shielding on Citric Acid Synthesis in Spleens of Irradiated Rats. Dose of fluoroacetate 3 mg/kg.

Dose of x-ray	Time of sacrifice (hr after x-ray)	Area irradiated	μg citric acid/g of spleen	
			Avg	Range
Control	—	None	1001	(968-1128)
800 r	24	Whole body	676	(480- 760)
800 r	24	Spleen only	1002	(856-1088)

TABLE II. Effect of Partial Shielding on DNA Phosphorus Content of Spleens of Irradiated Rats.

Dose of x-ray	Time of sacrifice (hr after x-ray)	Area irradiated	mg DNA phosphorus/100 g of spleen (wet wt) Avg	Range
Control	—	None	124	(111-137)
800 r	24	Total body	55	(50- 59)
800 r	24	Spleen only	98	(92-106)
800 r	72	Total body	67	(64- 75)
800 r	72	Spleen only	115	(97-124)

The histopathology of the spleen after 800 r of total body x-irradiation followed the pattern previously described by Murray(13). There was marked cellular destruction at 3 hours and phagocytosis and elimination of debris began at this time and progressed to completion within 24 hours. Further condensation of the spleen pulp was evident at 3 and 5 days with rare, medium and small lymphocytes remaining. When 800 r was given to the exteriorized spleen with lead shielding of the body there was some destruction of medium and small lymphocytes but fewer cells were altered than was the case after total body irradiation. More lymphocytes were present at 24 hours than at 12 hours after irradiation of the spleen alone and there was a continuous further increase in cellularity. Early germinal centers appeared in the lymphoid follicles at 3 days and almost complete recovery was observed at 5 days. Ectopic hematopoiesis showed a slight increase at 3 days and became prominent at 5 days. From a comparative standpoint the changes in the spleen following exposure of the exteriorized spleen to 800 r resembled those seen after 100 r of total body x-irradiation.

Discussion. The effects of lead shielding of various organs or areas of the body on the survival and the histologic changes after x-ray have been extensively investigated(14-21). The results of these previous studies, most of which have been done with mice, have led several investigators to postulate that the protection afforded by partial shielding is explainable on the basis of a humoral recovery factor(18,21), the destruction of a toxic factor(21), seeding of cells from shielded tissue(18,20) or temporary support of defense mechanisms(20). Major emphasis has been placed on possible factors(18,21) capable of accelerating recovery of hematopoietic tissues from radiation damage but thus far conclusive evidence in support of any of the possible explanations for modification of radiation injury by partial shielding is lacking.

The results of the present investigation have shown that 3 characteristic biochemical changes which occur in the spleens of rats after whole body irradiation are markedly reduced or prevented by body shielding although the radiant energy absorbed by the spleens is comparable in both cases. Histologic changes were generally in agreement with the biochemical findings. Evidence of rapidly reversible damage was seen in the spleens of body-shielded animals in contrast to the early and virtually complete destruction of the sensitive lymphoid and myeloid elements of the spleen within 12 to 24 hours after the same dose of total body x-irradiation. Failure of direct irradiation of the spleens of mice to cause atrophy comparable with that observed after whole body irradiation has recently been described(22) and a decreased mortality following regionally fractionated x-irradiation has been reported(23). These observations suggest that the susceptibility of the spleen to direct radiation injury is in some way dependent upon injury to other areas of the body.

The exact mechanism by which the spleen is protected by body shielding is not known. The data presented in this communication suggest the possibility that a substance essential for the normal metabolism of the spleen is supplied by the shielded tissue. According to this interpretation of the findings, the spleen is damaged only when the concentration of the essential factor falls below a critical level. Thus damage to the spleen would occur after whole body irradiation due to destruction of the factor *per se* or impairment of its production within the spleen and in other tissues. Preservation of an exogenous source of the material by body shielding during irradiation of the spleen might be responsible for the absence of marked biochemical changes in exteriorized, irradiated spleens. It is not established whether shielding the body during irradiation of the spleen prevents damage or accelerates recovery but the biochemical evidence supports the former view and further research on this possibility thus seems warranted.

That protection of the spleen by body shielding is not the result of detoxification of products formed in irradiated tissues was indicated by experiments in which the spleen and the remainder of the body were irradiated at separate intervals. After exposure of the body and the spleen at separate intervals of 12 hours the amount of damage to the spleen as measured by phosphatase assays was not appreciably different from that observed after total body x-irradiation.

Summary. Measurements of the adenosine triphosphatase activity, the 5-nucleotidase activity, the desoxyribonucleic acid concentration, citric acid synthesis and the histologic appearance of the spleens of rats given 800 r of whole body x-irradiation and the same dose of x-ray to the exteriorized spleens with the remainder of the body shielded were made. Shielding the body during irradiation of spleen prevented the large increases in the activity of phosphatases which occur in the spleen after whole body irradiation. Prevention of the inhibitory effect of x-ray on citrate synthesis and a much smaller decrease in desoxyribonucleic acid content of the spleen as well as less severe histologic changes were observed in the spleens of body shielded rats. The results of this study indicate that x-ray does not exert a direct effect on the enzymes involved in the biochemical reactions which were studied.

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Received February 11, 1955. P.S.E.B.M., 1955, v88.

Erythrocyte Destruction in Sickle-Cell Anemia: Simultaneous N^{15}-Hemin and N^{15}-Stercobilin Studies.* (21599)

G. Watson James, III[†] and Lynn D. Abbott, Jr.

From the Laboratory for Clinical Investigation, Department of Medicine and the Department of Biochemistry, Medical College of Virginia, Richmond.

Differential erythrocyte agglutination technics have demonstrated shortened peripheral existence of sickle-cell anemia erythrocytes transfused into healthy recipients or patients with sickle-cell anemia(2,3). Cells from different sickle-cell anemia patients may have different post-transfusion survival times, and available data suggest shorter survival of cells from donors with more severe anemia. Red cell destruction is probably closely related to the ease with which these cells undergo their characteristic distortion, and this might be the result of the very low solubility of reduced sickle-cell anemia ("S") hemoglobin(4). London, *et al.*(5) used the isotope method for labeling erythrocytes *in vivo* by oral administration of 41 g of approximately 32 atom % excess N^{15}-glycine(6) to a man with moderately severe sickle-cell anemia. This comparatively large amount of N^{15}-glycine was given over a two day period. Maximum erythrocyte labeling was obtained on 7th day with hemin N^{15} content greater than 1.1 atom % excess N^{15}. Random destruction of erythrocytes with a mean survival time of 42 days was indicated. This was in contrast to the fate of normal N^{15}-labeled red blood cells which leave the circulation as a function of their age(5,7,8). Excretion of isotope in stercobilin was not determined. Feeding of a large amount of N^{15}-glycine for two days prolongs the period of labeling of newly-formed erythrocytes by N^{15} from the "metabolic pool." Under these conditions labeling of heme continued for more than 20 days in normal individuals(5). In sickle-cell anemia the possibility of reutilization of N^{15} from cells containing high N^{15} concentrations undergoing random destruction is enhanced. Continued synthesis of labeled hemoglobin by red cells in sickle-cell anemia blood(9,10) also might be expected to take place to a greater extent with larger amounts of N^{15}-glycine fed over a longer period, although its effect on total synthesis of N^{15}-heme could no doubt be considered negligible(5).

The present experiment was designed to study red cell destruction and rate of hemoglobin turnover in a severe sickle-cell anemia patient using a small amount of N^{15}-glycine to minimize factors of prolonged labeling, reutilization, and peripheral incorporation. Previous studies in our laboratories demonstrated the practicability of using 1 g of N^{15}-glycine to label erythrocytes during a period of stimulated erythropoiesis(11). The N^{15}-concentration in fecal stercobilin, as in circulating heme, was determined to obtain additional information concerning destruction of labeled erythrocytes. Data on excretion of N^{15} in stercobilin in sickle-cell anemia have not been reported previously.

Experimental. A 21 year old, 120 lb, Negro male with severe sickle-cell anemia was fed 1 g of 31 atom % excess N^{15}-glycine in two 500 mg amounts at 11 a. m. and 3 p. m. without intervening lunch. Throughout the study he worked in our laboratory and was in a stabilized hematologic state as evidenced by his day to day course and serial hemograms. Hemoglobin of 6.0 g/100 ml and reticulocytosis of 23% indicated the severity of his disease. Previous electrophoretic studies had indicated 100% "S" type hemoglobin. Hemin was isolated(12) and recrystallized according to Fischer(13). Fecal stercobilin was extracted from *each* entire stool and was recrystallized after isolation (14). Purity was established by determination of optical rotation. Duplicate samples of hemin and stercobilin were converted to

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* An abstract has appeared(1). We are pleased to acknowledge the valuable technical assistance of Miss Mary Dodson, Mrs. Ruth Powell and Miss Vivian Wilkerson, and are very much indebted to Dr. William T. Ham and the Biophysics Department for use of the mass spectrometer.

[†] Markle Scholar in Medical Science.

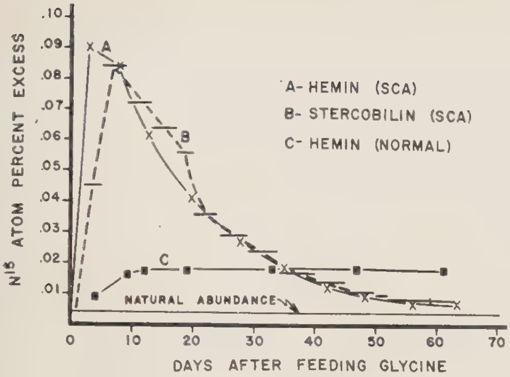

FIG. 1. N^{15}-hemin in sickle-cell anemia (A) and in a normal man (C), and N^{15}-stercobilin in sickle-cell anemia (B), after 1 g N^{15}-glycine. Horizontal bars indicate period representing each fecal sample.

nitrogen gas, and the N^{15} atom percent excess over tank nitrogen was determined for each sample in the mass spectrometer. Except for weekly withdrawal of 15-20 ml of venous blood for hemin analysis there was no extravascular blood loss, crisis, or any additional recognized clinical condition producing a random loss of red cells. Rate of red cell destruction was thus considered a measure of erythrocyte and hemoglobin formation.

Results. In Fig. 1, hemin curve (A) and stercobilin curve (B) present data expressed in atom excess N^{15} as the ordinate, and time in days as abscissa. Hemin curve (C) presents data obtained from a normal male of about the same weight who was fed 1 g of the same N^{15}-glycine under identical conditions. This curve is plotted for the first 60 days for comparison. In marked contrast to the normal subject, the sickle-cell anemia patient rapidly incorporated N^{15} into heme with maximum concentration found on 3rd day. The normal subject reached a much lower maximum about the 9th day, and then maintained a plateau. Since the N^{15} concentration (atom % excess) in circulating heme depends on amount of preexisting unlabeled hemoglobin with which newly-formed isotopically-labeled cells are diluted, and this is much smaller in the anemic individual, comparison of the extent of incorporation of N^{15} into heme with the normal in this case should be made in terms of absolute amount of N^{15} in heme. From determinations of total circulating hemoglobin in these subjects it was calculated that the absolute quantity of N^{15} incorporated into hemin in the sickle-cell subject was 1151 μg N^{15}, and in the normal, 288 μg N^{15}. Incorporation of N^{15} into heme in the sickle-cell subject was at least 4 times greater than in the normal and possibly more, since it is not certain that concentration on 3rd day was the highest value reached.

On the 8th day hemin N^{15} was lower than on the 3rd day. It is possible the N^{15} concentration was higher at some time between these two determinations. From the 8th day there was a rapid decline to the 56th day. Plotted on semi-logarithmic coordinates, hemin N^{15} disappearance curve from the 8th day was a straight line (Fig. 2). The apparently exponential disappearance of isotope from hemin is compatible with random destruction of erythrocytes, so rapid that any change produced by normal aging is obscured. Sheets, *et al.*(15), using inagglutinable cell count technics, indicated that such destruction would approach a pure logarithmic function. The cells cannot be given a definite life span, and it is more correct to consider their existence in terms of half-life, as determined below.

The N^{15}-stercobilin curve (B) was entirely different from the results of previously published studies with normal or pathologic subjects fed N^{15}-glycine(16-18), or infused with N^{15}-labeled red cells(19). Horizontal bars

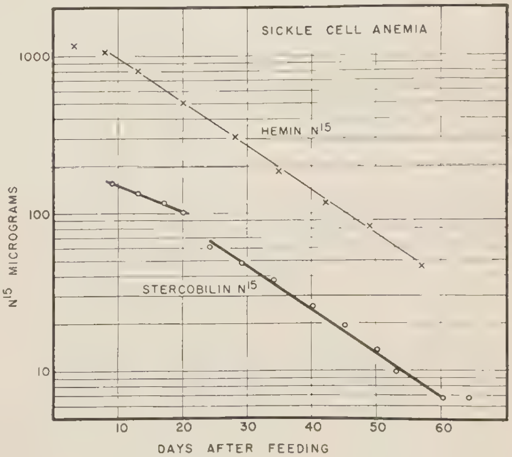

FIG. 2. Semilogarithmic plot of disappearance of N^{15} from hemin and stercobilin.

indicate number of days representing each fecal sample, since stools in this individual were usually four or five days apart. The stercobilin N^{15} concentration reached a maximum about the 7th day, after which there was a steady decrease to 19th day. After the 20th day a curvilinear disappearance of the isotope strikingly similar to the N^{15}-hemin disappearance curve was observed. The lag of the N^{15}-stercobilin curve behind the rising N^{15}-hemin curve, with isotope concentrations of both nearly equal at the peak of the stercobilin curve, is the particular sort of relation found to exist between time-course of labeling of a precursor and that of a product derived from it(20). The near coincidence of N^{15}-stercobilin and N^{15}-hemin curves in this case is consistent with an origin primarily from indiscriminant random destruction of circulating erythrocytes, labeled and unlabeled, having no definite life span.

Direct incorporation of isotope into stercobilin has been found to occur after feeding large amounts of N^{15}-glycine to normal man (16,17). The curve (B) prior to 20th day may represent, to an extent not known, the combined effect of this type of incorporation and the excretion curve of N^{15}-stercobilin derived from immediate, random destruction of labeled erythrocytes. Any initial labeling of stercobilin which might occur in this case would be obscured by labeled erythrocyte degradation, and could not be observed separately for study. Stercobilin labeling in the initial 10 to 20 day period after 1 g N^{15}-glycine has been observed by us in other individuals not having rapid red cell destruction(21).

For comparison of the rate of disappearance of isotope from hemin with that of fecal stercobilin, the absolute amounts of N^{15} in hemin (in micrograms) were calculated from the N^{15} concentrations (atom % excess above the natural abundance,(22) and the total circulating hemoglobin. Similarly, stercobilin N^{15} (μg/100 g feces) was calculated from the total fecal urobilinogen excretion (mean value, 1952 mg/100 g feces; 804 mg/day). Plotted on semi-logarithmic coordinates (Fig. 2), the data gave lines which were nearly parallel, indicating close correlation between rate of red cell destruction and rate of excretion of isotope in fecal stercobilin. From the N^{15} line (Fig. 2), or from a similar plot of the experimental data from Fig. 1, a half-life for erythrocytes of approximately 11 days was obtained. Using the equation for exponential disappearance ($C_t = C_o\ e^{-kt}$) a rate of red cell destruction and formation (rate of hemoglobin turnover) of 6.3%/day was calculated from the half-life. This turnover rate corresponds to a mean life or average time for erythrocytes in circulation of about 16 days.

Turnover rates also may be calculated directly from the data, *i.e.,* independent of the half-life determined by inspection of the logarithmic plots, by calculating the slopes for the straight lines by the least squares method from the natural logarithms of the N^{15} data. Turnover rates calculated in this manner were 6.37%/day (hemin data) and 6.3%/day (stercobilin data). These turnover rates give a calculated half-life of 11 days for erythrocytes, with a mean survival time of approximately 16 days.

There was no evidence of more than one population of cells. London, *et al.*(5) suggested the possibility of a small fraction of total red cell population with half-life time of approximately 7 days in their subject fed 41 g N^{15}-glycine. This was based on N^{15}-hemin data between 6th and 22nd days, a period during which labeling of cells is known to continue in normal individuals fed comparable amounts of N^{15}-glycine(5). It is difficult in this case to consider the N^{15}-hemin curve in the first 20 days as being other than the resultant of both labeling and destruction during this period. The continuing introduction of cells with a rapidly decreasing isotope concentration imposed on the random destruction curve could result in hemin N^{15} concentrations which would be not only higher than theoretical for the extrapolated disintegration curve, but with increments decreasing with time (greater slope). The deviation of N^{15} concentration from the theoretical destruction curve in the first 20 days in this case might be an indication of the rate of decrease of labeling rather than the result of disappearance of a shorter-lived population. The effect of prolonged labeling would be minimized with the 1 g dose used in the present study.

TABLE I. Comparative Data in Two Isotope Labeling Investigations in Sickle-Cell Anemia.

	Present study, 1 g glycine	London, *et al.*(5), 41 g glycine
Hemoglobin, g/100 ml	5.9	10.8
Red cell count, millions/mm^3	1.8	3.8
Hematocrit, %	16.0	31.0
Reticulocytes, %	22.8	10.2
Total circulating hemoglobin, g	385.0 (7 g/kg)	738.0 (11.7 g/kg)
Hemoglobin formation and destruction, g/day	24.2 (.44 g/kg/day)	17.7 (.28 g/kg/day)
Half-life, days	11	29
Mean survival time, days	16	42
Turn over rate, % per day	6.3	2.4

Although the turnover rate in our subject (6.3%/day) was 7-8 times normal (0.83%/day), the *rate* determined refers to the rate at which the individual was turning over *his* total mass of hemoglobin. With a total circulating hemoglobin of 385 g (Table I) daily synthesis amounted to 24.2 g (0.44 g/kg/day) about 4.3 times the normal 5.7 g/day expected in a 54.5 kg male. It was noted previously that the amount of isotope incorporated into heme was 4 times greater than in the control subject.

Crosby and Akeroyd(23) concluded that when erythrocyte life span becomes shorter than about 20 days the capacity of uninhibited marrow to compensate will be exceeded and anemia will result. In our patient, with a mean erythrocyte life of 16 days, erythrocyte production would have to be almost 8 times normal to prevent an anemia. It is evident that this was not done.

Hematologic severity of the disease may vary considerably in typical sickle-cell anemia patients. Comparison of our hematologic data with those of London, *et al.*(5) indicates the severity of anemia in our patient to be about twice that of theirs (Table I). Hemoglobin, red cell count, hematocrit and reticulocytosis differ by a factor approximately one-half or two. Random destruction with or without an accelerated senescence affects the time a cell survives in peripheral circulation. Observations in sickle cell-anemia patients having only "S" type hemoglobin and widely varying degrees of anemia suggest the possibility of other factors involved in hemolytic mechanism.

Substantial evidence for extra-corpuscular hemolytic mechanisms in sickle-cell anemia has not been found. Extra-corpuscular factors which might be present in the plasma or elsewhere in the donor and thus adding an additional hemolytic mechanism are excluded in the differential agglutination methodology where only washed red cells are transfused into the recipient. Even though the recipient may have the same fundamental intra-corpuscular erythrocyte defect, he might differ in degree of "hypothetical" extra-corpuscular influences. It is possible, therefore, that the severity of anemia in two different patients with only "S" type hemoglobin (small amounts of fetal hemoglobin excluded) may be the result of factors present in plasma of one and not present to the same degree in plasma of another. Perhaps rapid hemolysis in itself may give rise to a self-perpetuating cycle. Singer, *et al.* discuss variability of the life span of the sickle-cell erythrocyte, particularly with regard to the crises occurring in this disease(24). Isotopic study has the advantage of labeling and studying the cells in the same individual as well as circumventing changes in blood volume which might influence the results in agglutination methods.

Summary. 1. Study of erythrocyte destruction in sickle-cell anemia by analysis of hemin N^{15} disappearance and fecal stercobilin N^{15} appearance after a 1 g dose of N^{15}-glycine indicates a completely random destruction of circulating red cells. 2. This individual's cells had a half-life of 11 days indicating a turnover rate of 6.3% per day with a mean survival time of approximately 16 days. 3. There was no evidence of more than one population of cells. 4. Comparison with a similar study by London and coworkers shows a similar clinical condition but a physiological state twice as active, and further suggests still unknown factors involved in the destruction

of circulating red cells in this type of hemolytic anemia.

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Received February 11, 1955. P.S.E.B.M., 1955, v88.

Stimulation of Erythropoiesis in Irradiated Dogs and Rats. (21600)

FREDERICK STOHLMAN, JR., E. P. CRONKITE, AND G. BRECHER.

From National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, and Naval Medical Research Institute, Bethesda, Md.

Suppression of erythropoiesis by sublethal doses of whole body irradiation has been reported repeatedly. On the basis of histologic studies in mice(1,2) the normoblast was considered the most radiosensitive marrow cell. Radio-iron incorporation into red cells of the rat was markedly depressed by doses well below the lethal range(3). Using recovery of the normal red cell mass following bleeding as a measure of erythropoiesis, suppression of erythropoiesis in cats given sublethal doses of X-ray could not be demonstrated(4). These apparently contradictory observations might be explained by species variations, or the hypoxia incident to bleeding may influence the recovery of erythropoiesis following x-irradiation. Evidence to support the latter will be presented in this report.

Materials and method. Twelve dogs weighing 10 to 15 kilos were divided into 4 groups: one bled immediately prior to irradiation; a second bled immediately following irradiation; a third bled but not irradiated; and the fourth irradiated but not bled. The irradiated animals received 150 r total body irradiation from a Co^{60} source in 4 π geometry (5). In the animals which were bled, 30% of the blood volume, calculated as 8% of the body weight, was removed. Erythropoiesis was estimated from the hematocrit and reticulocyte levels, performed three times a week, and from the determination of iron incorporation into red cells. For the latter, 5 to 10 μc Fe^{59} bound to the iron binding globulin were injected intravenously on the 5th post-treatment day. This material was prepared by incubating homologous heparinized plasma with Fe^{59} as $FeCl_3$ for 60 minutes at room temperature. Approximately 0.25 to 0.4 μg of metal containing 0.8 to 1.2 μc of Fe^{59} were added per ml of plasma. Following I.V. injection of Fe^{59}, 1 to 3 ml samples of venous blood were taken daily for the first 4 days and on alternate days, through the 16th day. After the 1st day, the plasma contained insignificant amounts of radio-iron, so that washing of the red cells was unnecessary. The red cells were hemolyzed with saponin and the volumes brought to 6 cc with distilled water. Activity was determined in a shielded well-type scintillation counter, with a background of 200 CPM. 12,800 counts were measured in duplicate. Total Fe^{59} in circulating red cells was estimated on the basis of a computed whole blood volume of 8% of body weight. The usual assumption of a constant red cell mass which is implied in computing activity in terms of packed cells, could not be made in the present experiment. The blood volume, however, is known to be reasonably constant after low doses of irradiation(6). Bleeding did not appear to affect the blood volume markedly, as judged from the drop in hematocrits. For graphic representation, Fe^{59} incorporation was expressed in percent of the injected dose. In a similar experiment in *rats*, females of the Sprague-Dawley strain, weighing 145 to 180 g, were used. Thirty rats were randomly assigned to 5 groups. All rats received 200 r (Co^{60} source). One group was bled by cardiac puncture immediately prior to irradiation, 25% of the blood volume, computed as 7% of body weight, being removed. A second group was bled immediately after irradiation, and a third 24 hours after irradiation. A fourth group received 0.3 cc/100 g of a 1% para-aminopropiophenone (PAPP) solution in propylene glycol immediately after irradiation. The fifth group served as irradiated controls. 0.3 cc of Fe^{59} tagged plasma, prepared as described above, was injected I.V. on the second post-irradiation day. Blood samples were removed by cardiac puncture 48 hours later, the red cells were washed once, and the activity determined as described. 12,800 to 64,000 counts were measured in duplicate. Reticulocyte counts were made on aliquots of the same samples. Iron incorporation in non-irradiated rats was determined in separate experiments.

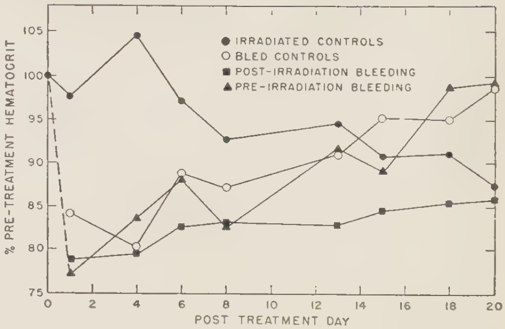

FIG. 1. Effect of pre- and post-irradiation phlebotomy on hematocrit in the dog. Avg values for each group expressed as percent of pretreatment hematocrit.

Results. The reticulocyte values of the irradiated dogs decreased from control levels of 0.2 - 0.6% to less than 0.1%. Beginning on the 13th-15th post-irradiation day the reticulocytes slowly increased to pre-irradiation values. In the animals bled either before or after irradiation there was an initial fall in reticulocytes followed by a significant reticulocytosis of 0.5-3.1% beginning by the 4th day in all but one in which reticulocytosis did not occur until the 8th day.

Hematocrits of irradiated dogs that had not been bled fell gradually and reached 85% of pre-irradiation levels by the 20th post-irradiation day (Fig. 1). This fall could be

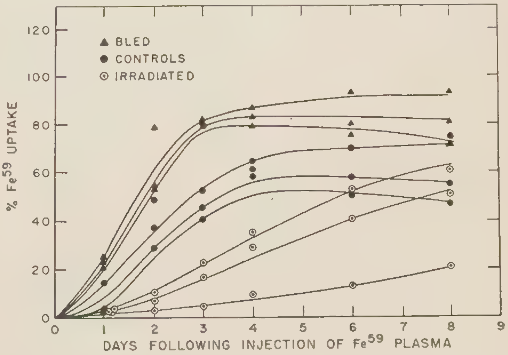

FIG. 2. Fe^{59} incorporation in normal, bled, and irradiated dogs. Note rate of appearance of Fe^{59}. In normals, a plateau was reached at 4 days; in bled dogs, at 3 days. Contrast with slow steady rise over 8 days in irradiated dogs.

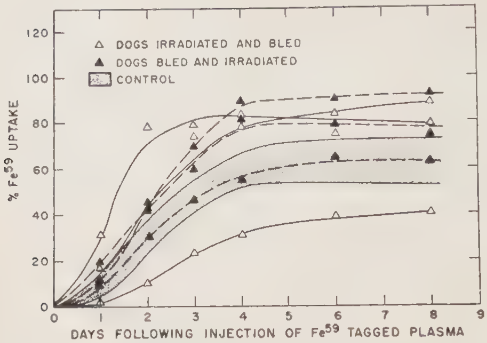

FIG. 3. Fe^{59} incorporation in dogs bled immediately before and after irradiation. Shaded areas indicate range of 3 normal dogs.

accounted for by suppression of red cell production in the presence of a constant total blood volume. In the remaining dogs, bleeding reduced the hematocrits initially by 20-25%. In the bled controls and those bled immediately prior to irradiation the hematocrits gradually rose and reached pre-bleeding levels by the 20th day. In those bled following irradiation there was a more gradual rise and pre-bleeding levels were not attained by the 20th day.

Iron incorporation in 3 groups of dogs is shown in Fig. 2. Following injection of radio-iron into normal dogs, the amount of iron incorporated into red cells was nearly at its peak in the peripheral blood by the 4th day. During the 5th to 8th day, radio-iron increased only slightly or not at all. In dogs that were bled but not irradiated, iron incorporation was more rapid. A plateau of radio-iron in the peripheral blood was reached by the 3rd day, with little further increase thereafter. In irradiated dogs, radio-iron increased more slowly than in controls, but at a steady rate without any change in slope of the iron incorporation curves during the first 8 days after injection. The linear increase over this period was in contrast to the S-shaped curves of both the bled and normal controls. Accelerated erythropoiesis in the bled controls and suppression of erythropoiesis in the irradiated dogs is evident. Since the 8 day values for the 3 groups overlap, it is apparent that the 24 to 72 hour values and the shape of the curves are better indicators of red cell production than maximum incorporation.

Iron incorporation curves for dogs bled before or after irradiation are shown in Fig. 3. Five of the curves resemble those of normal dogs reaching a plateau on the 4th day. They differ markedly from the linear increments of iron incorporation of irradiated controls (Fig. 2). One dog bled after irradiation behaved like the bled, non-irradiated group in that the plateau of the curve was reached on the 3rd day. This dog had an elevated reticulocyte count of 1.1% before irradiation, indicating an increased erythropoiesis of some duration. In retrospect, this dog should not have been used in the experiment since erythroid hyperplasia reported to protect the erythropoietic system from radiation injury(7) may have been present. The reticulocyte values (4.5%) and hematocrit rise from this animal have not been included.

In contrast to the continued rise in hematocrit and reticulocytosis in the bled, irradiated animals, the white cell and platelets fell progressively to reach minimum values between the 13th and 18th days. The minimum values of 8-26,000 platelets and 1800-3500 white cells in irradiated controls were slightly lower than those in animals bled either before or after irradiation in whom the minimum platelet values ranged from 20-42,000 and white cells from 3,300 to 6,700.

The effects of pre- and post-irradiation bleeding and of post-irradiation administration of PAPP on iron incorporation into red cells of irradiated rats are illustrated in Fig.

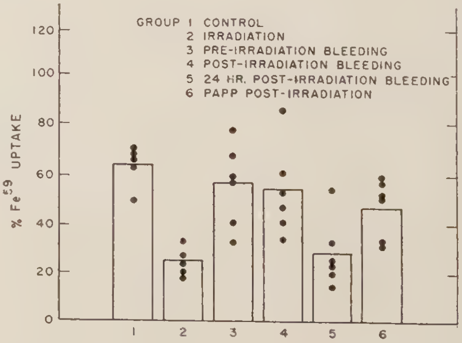

FIG. 4. Effect of hypoxia before and after irradiation on erythropoiesis as measured by Fe^{59} incorporation. Fe^{59} injected 48 hr after irradiation and percent incorporation in red cells measured 48 hr after injection.

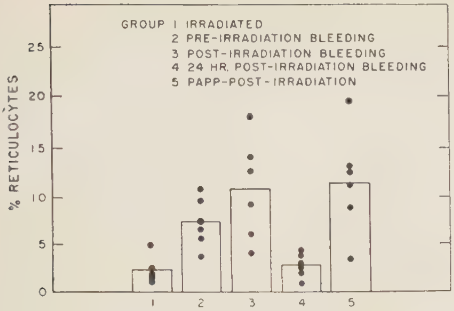

FIG. 5. Reticulocyte values 96 hr after irradiation in rats with pre- and post-irradiation hypoxia.

4. All but one of the irradiated control rats incorporated less than 31% of injected Fe^{59} in 48 hours. Acute blood loss, either immediately before or after irradiation, increased iron incorporation in each of 6 rats to above 30%. The percent of injected iron incorporated in 48 hours by normal rats is slightly higher, varying from 50 to 75%, with a mean of 66%. PAPP was about as effective as immediate bleeding in increasing iron incorporation. Bleeding 24 hours after irradiation had no appreciable effect, five of six animals showing iron incorporations below 34%.

Reticulocyte counts (Fig. 5) in 5 of 6 control irradiated rats were below 3% on the 4th post-irradiation day. One had a reticulocyte count of 4.7%. Marked reticulocytosis (Fig. 5) was present in rats bled immediately before or after irradiation, and in those given PAPP immediately after irradiation. Reticulocyte counts in rats bled 24 hours after irradiation remained below 4%. Normal rats of this strain in our laboratory have reticulocyte counts of 3-7%. Reticulocytes in the rats that were both bled and irradiated, were in most instances above normal while the iron incorporation was within the normal range and in a few below normal. However, iron incorporation data reflect erythropoietic activity primarily on the second day, since Fe^{59} is rapidly cleared and presumably quickly incorporated into red cell precursors, though maturation and appearance of tagged red cells in the circulation require 24 to 48 hours. The reticulocyte levels were determined on the fourth day and are therefore not necessarily indicative of red cell production on the second day alone. Reticulocyte levels are also influenced by accelerated maturation or release of immature cells, and may therefore be higher than expected from actual production rates.

Discussion. The results confirm previous reports of a significant depression of erythropoiesis by sublethal doses of irradiation. Valentine's(4) apparently contradictory observation that sublethally irradiated animals may recover from an anemia induced by pre-irradiation blood loss as rapidly as a bled non-irradiated animal could be confirmed. This observation can now be interpreted as a reversal or modification by the acute blood loss of the usual radiation injury to erythropoiesis. This interpretation is supported by the observation that red cell production, though ordinarily suppressed in irradiated dogs and rats, can be accelerated to normal levels by post-irradiation as well as pre-irradiation bleeding and, in rats, by the administration of PAPP after irradiation. These results could be due to inhibition of the destruction of erythropoietic cells known to occur following irradiation, or due to accelerated regeneration. This accelerated regeneration, however, appears to be restricted to the erythropoietic system and can no longer be induced by blood loss 24 hours after exposure to X-ray.

Since PAPP produces a severe, though transient, hypoxia it is tempting to speculate that the common denominator of the action of PAPP and bleeding is hypoxia which elicits the release of a humoral factor responsible for stimulation of erythropoiesis. Evidence for the existence of such a factor or factors has been demonstrated recently(8-10) and the isolation of one factor in crude form claimed (11).

Summary. Following whole body exposure to sublethal doses of X-rays, erythropoiesis was depressed in both dogs and rats, but could be markedly increased by bleeding the animals shortly before or after irradiation. In rats, post-irradiation administration of PAPP resulted in a similar increase in erythropoiesis. Blood loss 24 hours after irradiation had no appreciable effect. The data indicate the reversibility or modification of injury to the erythropoietic system without similarly

affecting the recovery of other bone marrow elements.

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Received February 16, 1955. P.S.E.B.M., 1955, v88.

Relationship Between Vitamin E and Dietary Minerals in Mouse "Paralysis".* (21601)

Joseph T. King, Y. Chiung Puh Lee, and M. B. Visscher.
From the Department of Physiology, University of Minnesota, Minneapolis.

The vit. E requirements and the manifestations of deficiency have been reported for several species. One result of E deficiency is the "paralysis" of suckling rats born to E-deficient mothers reported by Evans and Burr(1). Later Ringsted(2) observed the disorder in adult rats kept on chronic E deficiency. Similar results were reported by Burr, Brown, and Moseley(3). Studies of the mouse on E-deficient diets such as those used for rats have yielded conflicting results. Bryan and Mason(4), reported that suckling mice born to E-deficient mothers (receiving the usual single dose of the vitamin early in pregnancy) showed no "paralysis". Goettsch (5) observed no paralysis in either sucklings or older mice. Pappenheimer(6) reported that these mice showed the degenerative lesions of skeletal muscle expected in E deficiency in spite of the fact that there were no symptoms of paralysis. Menschik *et al.*(7) using the British M.R.C. strain of mice observed typical symptoms in animals beyond 6 months of age, the maximum incidence falling between 12 and 18 months. Tobin(8) failed to get paralysis using the ordinary diet containing 2% cod liver oil but did observe up to 17% of mice showing symptoms on diets containing 10% or 20% cod liver oil.

We have observed that the mineral content of the diet is an important factor in respect to the development of the symptoms of "paralysis" in the adult mouse. This is a report on certain phases of the study.

Materials and methods. C_3H male mice were started on the diets at 21-23 days of age. They were housed in pairs on shavings in cages made of sheet metal and screen. Tap water was continuously available. They were fed *ad libitum* from metal cups. Room temperature was 80° ± 2°F. The mice and food cups were weighed and the cups changed weekly. The following salt formulas were used. *Salts A*: (Modification of mixture 351,

TABLE I. Growth Rate on Diets Used; Average Group Weight in Grams.

	Diets:				
Age, wk	1	2	3	4	5
3	8.5	8.6	9.4	10.5	10.4
5	20.3	20.4	21.1	18.7	20.0
10	28.5	27.8	27.6	26.7	26.4
15	32.7	30.4	33.2	30.9	28.8
20	34.8	31.2	35.2	32.3	29.8
25	37.7	31.6	38.5	35.1	30.9
35	41.0	32.3	40.9	36.8	31.0
45	40.7	32.3	41.8	37.6	30.9

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* This investigation was supported in part by research grant from National Heart Institute, of the National Institutes of Health, Public Health Service.

TABLE II. Showing Time of Onset and Incidence of "Paralysis" on Various Diets.

	Diets: 1	2	3	4	5
First noted		6 mo			6 mo
% mice involved†	0	100	0	0	100
Mortality, 52 wk	0/24	3/24	2/20*	4/24*	8/24

* Death from causes other than "paralysis."

† By 8 mo of age.

Hubbell, Mendel, and Wakeman, *J. Nutrition*, 1937, v14. 273). $CaCO_3$, 544; $MgCO_3$, 25; $MgSO_4$, 16; NaCl, 69 (iodized table salt, used to avoid difference in this respect from salt C); KCl, 112; KH_2PO_4, 212; $FePO_4$, 21 ("Ferric Phosphate soluble" N.F. VIII, powder). *B*: Same as *A* except ferrous sulfate used instead of ferric phosphate. *C*: (a modification of mixture 12, Jones and Foster, *J. Nutrition,* 1942, v24 245). NaCl, 292.5 (iodized table salt used to reduce tendency to liberate iodine); KH_2PO_4, 816.6; $MgSO_4$, 120.3; $CaCO_3$, 800.8; $FeSO_4 \cdot 7\ H_2O$, 56.6; KI, 1.66; $MnSO_4 \cdot 2\ H_2O$, 9.35; $ZnCl_2$, 0.55; $CuSO_4 \cdot 5\ H_2O$, 1.00; $CoCl_2 \cdot 6\ H_2O$, 0.05.

Diets #1: Glucose, 33; casein, 28; lard, 17; yeast, dried brewers, 8; alfalfa leaf, dried, 4; salts A, 7; cod liver oil, 2; wheat germ oil, 1. #2: Same but without wheat germ oil. #3: Same as #1 except salts C, 4%. #4: Same as #3 except without wheat germ oil. #5: Same as #1 except with salts B.

Results. Growth rates on various diets are shown in Table I. Data on development of "paralysis" are shown in Table II.

The first symptom noted in animals, which eventually become characteristically involved, is a slight, intermittent ataxia. This defect is progressive. Later the animals show a slight tendency to walk with the hind legs spread out. In the fully developed stage they seem completely unable to control the hind legs in this respect. In this stage the rear end of the body is being dragged. Even in the very late stage, however, it appears that loss of control is not absolute since ineffective efforts to use the hind limbs may be noted.

Loss of tone in the involved limbs is evident. There is probably some loss of skin sensitivity. Patchy loss of hair occurs irregularly chiefly over the ventral surface of the body (limited to the abdomen). There are no obvious abnormalities in the head region. We have not observed involvement of the front legs. No trophic ulcers have been noted. The general appearance of the mice does not suggest atrophy of the muscles.

Mice may live for months in this condition. Immediate cause of death is not evident at autopsy. The muscles do not appear to be atrophied. There may be patchy involvement of the lungs. Some pigmentation of the central nervous system is evident.

Discussion. The above results leave no doubt that mice develop typical symptoms of "paralysis" when fed suitable diets. The symptom complex is very similar to that described for the rat by Ringsted(2) and later by Burr, Brown, and Moseley(3).

It is not clear why some investigators working with mice have failed to obtain positive results. Tobin(8) did not observe paralysis in mice on diets containing the usual 2% of cod liver oil but did observe the symptoms when amount of oil was increased to 10% or 20%. Pappenheimer(6) reported pathological findings in mice from experiments described by Goettsch(5). Microscopic lesions without paralysis were present in skeletal muscle in about 20% (average for various age groups) of mice up to 439 days of age. Menschik(7) *et al.* have described symptoms of paralysis seen in the British M.R.C. strain of mice subjected to chronic vit. E deficiency. The average age of onset was 403 days (209-648 days). The slowly progressive character of the disorder, as they observed it, is indicated by the fact that one mouse lived 262 days after onset of dystrophy. Their description of symptoms and their photographs leave no doubt that in all essential respects the disorder is the same as that observed by us.

It is known that certain dietary factors influence the results of E deficiency. Mackenzie(9) found that deficiency of vit. A, pyridoxine or protein intensified the changes due to E deficiency in the rat. Mackenzie(9), Tobin(8) and Rumery(10), and McCay and coworkers(11,12) have reported that cod liver oil increases the severity of E deficiency in a number of species.

The data reported above show that the mineral content of the diet is a determining factor in the development of mouse "paralysis."

Our results cannot be explained on the assumption that we are dealing with a simple vit. E deficiency uncomplicated by other factors. Such an assumption would be reasonable if we had only the result of removing wheat germ oil from diet #1. In this case removal of oil results in animals developing the deficiency syndrome which does not occur when the diet contains the oil. However, removal of oil from diet #3 fails to produce the same result. During recent years several groups of mice have lived their whole life span on this diet without showing such symptoms of paralysis. The difference between diets 1 and 3 is that the latter is made with a more complex salt mixture containing trace elements not found in salt formula A.

The complexity of the problem is further increased by the fact that when we substitute ferrous sulfate (the usual iron salt in formula C) into formula A, instead of ferric phosphate the mice develop the "paralysis" syndrome *even though diet contains the usual amount of wheat germ oil.* Thus diet 5 produces the same symptoms as diet 2 except that the symptoms are somewhat worse and mortality at 52 weeks is greater. It is tempting to assume that the trace elements in the modified Jones-Foster formula (but lacking in the modified Osborne-Mendel formula) determine the effect of ferrous sulfate. However, there are other differences between these salt formulas. At present the reason for the observed difference is not known. We also have no convincing explanation for the fact that removing wheat germ oil from one diet produces "paralysis" which is not seen when the oil is removed from the diet made with the modified Jones-Foster formula.

As we reported earlier(13), C_3H mice regularly die of heart failure beyond one year of age when fed the diet made with the modified Jones-Foster formula (diet #3 above). Whether there is a relationship between the observed heart failure and the "paralysis" syndrome remains to be determined. It is possible that they are different manifestations of chronic E deficiency.

Since salts have been found to play an important role in determining the response to vit. E, we point out that a somewhat similar situation has recently been reported for certain B vitamins. Layman and Elvehjem(14) called attention to the lability of thiamine in certain purified rations. They found that as much as 80% of thiamine could be destroyed in 2 weeks. Rombouts(15) reported that the destructive effect of the Phillips and Hart salt mixture on B vitamins was not observed when a U.S.P. mixture was used. Waibel, Bird, and Baumann(16) found thiamine to be much less stable in a diet containing salts than in a salt free diet and that a ballmilled salt mixture was more destructive than a mixture of ordinary fineness. They discuss possible explanations.

For a detailed consideration of the relation of vit. E to degeneration of muscle in various species the reader is referred to the comprehensive review of Mackenzie(9).

Summary. 1. Typical symptoms of "paralysis" occur in C_3H male mice when wheat germ oil is removed from the diet made with a modified Osborne-Mendel salt mixture but not when the diet is made with a modified Jones-Foster mixture. 2. The same symptoms develop when ferric iron is replaced by ferrous iron in the modified Osborne-Mendel mixture even though wheat germ oil is present in the diet. 3. Although the observed "paralysis" is apparently identical with that seen by several investigators in other species of mammal in association with vitamin E deficiency, the fact that the mineral content of the diet determines the character of the manifestations of such vitamin deficiency raises questions concerning the mechanism of the effects which are still unanswered.

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Received February 2, 1955. P.S.E.B.M., 1955, v88.

Carbohydrate Metabolism in Experimental Salicylism.* (21602)

ROBERT W. WINTERS† AND MARY F. MORRILL. (Introduced by Edward C. Curnen.)

From Department of Pediatrics, University of California Hospital, San Francisco.

Recent experimental work has indicated that large doses of salicylates exert a marked influence upon the carbohydrate metabolism of the experimental animal(1-10). In this paper, we wish to present further observations dealing with these effects. Such information may have relevance to an understanding of the profound metabolic disturbances which occur in clinical salicylate intoxication and may provide further insight into the pharmacology of this group of drugs.

Methods. Male rats of the Sprague-Dawley strain weighing between 200 and 300 g were used. In all experiments, food was withdrawn 24 hours prior to the administration of drugs. Water was allowed throughout the course of the experiments. *Sodium salicylate* was administered subcutaneously in a peanut oil suspension; control rats received peanut oil alone in comparable volumes. Compound F alcohol‡ in saline suspension was also administered subcutaneously. *Chemical methods* were as follows: blood urea nitrogen (BUN), Conway(11); urinary nitrogen, micro-Kjeldahl; liver and muscle glycogen, Pfluger digestion with estimation of the reducing substance following hydrolysis by the Nelson method(12). Free flowing tail blood was used for all blood chemical determinations. For the glycogen determinations, the animals were anesthetized with nembutal and the gastrocnemius muscle and the liver rapidly removed and immediately placed into tared stoppered vessels containing 30% KOH. In calculating the *nitrogen balances*, correction was made for the rise in BUN of the salicylate-treated rats by subtracting the BUN at the beginning of the experiment from that at the end and multiplying the difference by an estimated volume distribution of urea assumed to be 60% of the body weight at the end of the experiment. This figure was then added to the urinary nitrogen. Such a correction is essential for the accurate determination of the nitrogen balance, since salicylate-treated animals frequently show a significant rise in BUN; this rise is probably secondary to electrolyte and water deficits as a result of the rapid excretion of these substances under the influence of toxic doses of salicylates (13).

Results. Liver Glycogen: Table I shows the data obtained on the levels of liver glycogen in rats treated in various ways. All animals were fasted 24 hours in order to deplete the liver of preformed glycogen (Group O). During the next 24 hour period, the control group (Group I) showed some increase in the

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* This investigation received support from the Jefferson MacLeod Moore Estate of University of California Medical School.

† Bank of America-Giannini Foundation Fellow in Pediatrics. Present address: Department of Pediatrics, University of N. Carolina School of Medicine, Chapel Hill, N. C.

‡ Compound F alcohol was provided by Dr. Peter H. Forsham from material furnished by Merck and Co.

TABLE I. Liver Glycogen Levels.

Group	Treatment*	No. of animals	Time of salicylate admin., hr	Mean and S.D. (mg % wet wt)
0	24 hr fast	6	—	45 ± 26
I	Peanut oil	5	—	249 ± 53
II	75 mg Na Sal.	5	0	113 ± 87
III	10 mg F alc.	6	—	3230 ± 318†
IV	75 mg Na Sal. + 10 mg F alc.	7	0	351 ± 180
V	25 mg Na Sal. + 10 mg F alc.	5	0	3610 ± 340†
VI	75 mg Na Sal. + 10 mg F alc.	5	12	380 ± 299
VII	25 mg Na Sal. + 10 mg F alc.	4	12	1195 ± 301†‡

* All drug doses in mg/100 g body wt. All animals fasted 24 hr before any drugs given. Compound F alcohol given at this time in all cases (0 hr).

† Indicates statistical significance at a level of $p = <0.01$ when compared to Group I.

‡ Indicates statistical significance at a level of $p = <0.01$ when compared to Group V.

liver glycogen; treatment of a comparable group of animals with a large dose of sodium salicylate tended to depress this rise (Group II), but this difference was not statistically significant.

Treatment with *large doses* of Compound F alcohol alone (Group III) produced considerable deposition of glycogen as expected, but when both Compound F alcohol and salicylate were administered together (Group IV), no such rise occurred. The differences between Group III and Group IV were highly significant. A smaller dose of salicylate (25 mg/100 g) was ineffective in preventing the glycogen deposition induced by treatment with steroid (Group V) when both agents were administered at the same time. It seemed likely that this lack of effect with the smaller dose might be due to the relatively slow absorption of the steroid in relation to the more rapid absorption of the salicylate. Indeed, measurement of blood levels after various doses of salicylate revealed that with the 25 mg/100 g dose, the major rise and fall in the blood level occurred in the first 12 hours whereas this rise and fall occupied nearly 24 hours with the 75 mg/100 g dose.

By *spacing injection* of salicylate 12 hours following the dose of steroid, the glycotropic effect of Compound F alcohol was antagonized with both dose levels (Groups VI and VII). These experiments demonstrate that the effects being studied were not confined to large nearly fatal doses, but could also be obtained with smaller, though still toxic, doses.

Nitrogen Balances: Table II summarizes data obtained on the nitrogen balances. Treatment with either Compound F alcohol or salicylate (75 mg/100 g) produced a greater negative nitrogen balance than that of the control. This effect was not additive when both agents were used concurrently.

Muscle Glycogen: These data are also shown in Table II. Salicylate treatment (75 mg/100 g) produced a considerable fall in the muscle glycogen, an effect which was countered by simultaneous administration of Compound F alcohol.

Discussion. The inhibition of the glycotropic effect of Compound F by salicylates demonstrated in the rat liver in this paper may be related to the findings of other workers that salicylate causes a breakdown of preexisting liver glycogen in the normal rat, the alloxan-diabetic rat and the adrenalectomized rat(1,5-7) as well as being related to the demonstration of a block in the deposition of liver glycogen in the alloxan-diabetic rat, the cortisone-treated adrenalectomized rat and the normal rat receiving a glucose load by mouth(1,5-8). Both of these effects of salicylate, acceleration of glycogenolysis as well as inhibition of glycogenesis under the influence of adrenal steroids, have been confirmed in an *in vitro* system by M.J.H. Smith(14). As judged from the magnitude of the nitrogen balances reported in this paper, the failure to deposit liver glycogen in the salicylate-steroid treated group cannot be attributable to failure of provision of adequate precursors of glycogen from protein. Rather it would seem that such precursors follow other metabolic pathways, possibly conversion to fat as well as to blood glucose.

From previous work, salicylate is known to exert other effects upon the metabolism of carbohydrate in the general direction of increasing utilization of carbohydrate in the peripheral tissues. Thus large doses of these drugs have been found to reduce the degree

TABLE II. Nitrogen Balances and Muscle Glycogen Levels.

		Nitrogen balance		Muscle glycogen	
Group	Treatment*	No. of animals	Mean and S.D. (mg/rat)	No. of animals	Mean and S.D. (mg % wet wt)
I	Peanut oil	5	−219 ± 45	4	562 ± 48
II	75 mg Na Sal.	5	−365 ± 35†	6	154 ± 44†
III	10 " F alc.	5	−438 ± 56†	6	436 ± 94‡
IV	75 " Na Sal. + 10 mg F alc.	5	−398 ± 29†	5	550 ± 53

* All drug doses in mg/100 g body wt, administered after a 24 hr fast.
† Indicates statistical significance at a level of $p = <0.01$ when compared to Group I.
‡ *Idem* Group II.

of glycosuria, to lower the blood sugar, and to improve the glucose tolerance in various forms of experimental diabetes(1-7). Such effects, moreover, occur with either no change or even a rise in the urinary nitrogen excretion(3,7). It is possible that the lowering of muscle glycogen recorded in this paper represents another manifestation of this acceleration of carbohydrate utilization.

Our data as well as those of others reviewed above demonstrate certain antagonistic effects of salicylate and adrenal steroids in carbohydrate metabolism. These findings have bearing on recent speculation that high doses of salicylate activate the anterior pituitary-adrenal cortical axis to a heightened rate of secretion. Such speculation derives primarily from a similarity of the therapeutic action of these two agents as well as from the findings suggesting adrenal cortical stimulation in experimental or clinical salicylism(13,15,16).

If large doses of salicylate do cause increased secretion of the adrenal cortex, it is apparent that the excessive adrenal steroid output does not exert its "pure" effects upon the carbohydrate metabolism of the animal. Rather these effects are markedly conditioned directly or indirectly by the presence of salicylate so that the resultant pattern of carbohydrate (and electrolyte(13)) metabolism is distinctively different from that seen with "pure" adrenal cortical stimulation.

Summary. Fasting rats given large doses of sodium salicylate fail to deposit liver glycogen even when they are given a major stimulus for such deposition in the form of Compound F. Yet such animals continue to break down body protein at an accelerated rate as judged by the nitrogen balances. The level of muscle glycogen salicylate-treated rats is reduced and this effect is countered by concurrent treatment with Compound F. The significance of these findings is briefly discussed.

The authors wish to express their appreciation to Professor William C. Deamer and to Dr. Peter H. Forsham for advice and encouragement and to Dr. Forsham for making the facilities of the Metabolic Unit of the School of Medicine available.

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Received February 3, 1955. P.S.E.B.M., 1955, v88.

Effect of Whole-Body X-Irradiation on Concentrations of DPN and DPNH in Rat Liver.* (21603)

HERBERT J. EICHEL AND M. A. SPIRTES. (Introduced by M. J. Boyd.)

From Divisions of Biological Chemistry and Pharmacology, Hahnemann Medical College, Philadelphia, Pa.

Swallow(1) observed that DPNH was not produced in solutions of DPN irradiated with 30,000 or 120,000 r of γ-rays; however, DPN solutions irradiated in the presence of ethyl alcohol gave rise to DPNH. He concluded that the free C_2H_4OH radical was responsible for this reaction. While the investigation described in the present paper was in progress, Barron *et al.*(2) confirmed that DPN was not reduced by X-irradiation (100,000 r) but showed that DPNH was readily oxidized (1,000 r). A decrease in absorption spectrum of DPN at 260 mμ, corresponding to the pyrimidine moiety of the coenzyme, was found(2). DPN reduced by X-irradiation in the presence of alcohol or lactate was reduced to a compound which was only partially active when tested with crystalline alcohol dehydrogenase(2). Since DPN is an essential component of a number of anaerobic dehydrogenases, it was considered of interest to determine whether whole-body irradiation has any effect on this coenzyme. Under normal conditions, both DPN and DPNH are present in tissues. Conceivably, either of these compounds could be chemically altered by direct ionization or by the products of irradiated cellular water or both. Such changes would escape detection in the usual manometric and spectrophotometric assays for DPN-linked dehydrogenases because these technics require the fortification of homogenates with large amounts of purified DPN. Hence, while a change in dehydrogenase activity employing optimal *in vitro* conditions may be clearly interpreted as an alteration in the apoenzyme component of a pyridinoprotein, unchanged enzyme activity may still involve significantly lowered *in vivo* nucleotide levels possibly masked by the addition of pure coenzyme.

The experiments presented here show clearly that the concentration of oxidized DPN is relatively unaffected in the livers of rats exposed to a lethal dose of whole-body X-irradiation, while that of the reduced coenzyme is significantly lowered.

Methods. Male rats (80-120 g) obtained from the Wistar Institute were irradiated as previously described(3) with a total dose of either 700 or 980 r of 240 KV X-rays filtered through 0.25 mm Cu and 1.0 mm Al at an average rate of 121.8 r/minute and a target distance of 50 cm. For the first 24 hours after irradiation, food was withheld from irradiated and control rats. A stock commercial diet (Nurishmix or Purina dog chow checkers) was then pair-fed to all animals. Control and X-rayed rats were sacrificed by decapitation. Portions of the livers were removed and blotted free of blood, and DPN and DPNH determinations were carried out according to a method reported recently(4). The method involves heat-treatment (100° C) of liver suspended in buffer followed by homogenization, further heating, and high-speed centrifugation to clarify the extract. Both forms of the coenzyme are determined spectrophotometrically on separate aliquots of one extract using crystalline alcohol dehydrogenase and either ethanol and buffer at pH 9.0 (for DPN) or acetaldehyde and buffer at pH 7.1 (for DPNH). Since it has been shown (4) that liver samples analyzed several minutes after decapitation contain significantly less DPN than samples taken as soon after sacrifice as possible, it was deemed of interest to test samples from all livers for DPN at both time intervals. If the irradiation had effected a marked change in the activities of either DPN nucleosidase or pyrophosphatase, this procedure might make possible the detection of an increased breakdown of coenzyme

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* The following abbreviations are employed: DPN = oxidized diphosphopyridine nucleotide; DPNH = reduced diphosphopyridine nucleotide.

This study was supported by grants from the U. S. Atomic Energy Commission and the U. S. Public Health Service.

TABLE I. Effect of Whole-Body X-Irradiation on the Concentrations of DPN and DPNH in Rat Liver. All concentrations are expressed on basis of 10 mg N.

Exp. No.	r	Group	No. of rats	μg DPN			μg DPNH			μg DPN + DPNH			DPN/DPNH		
				Avg*	1.5	4	Avg	1.5	4	Avg	1.5	4	Avg	1.5	4
1	700	Control	10	108†	117	99	80	80	80	188	197	179	1.35	1.46	1.24
		X-ray	16	116	116	116	77	77	77	193	193	193	1.57	1.57	1.57
2	980	Control	6	101	107	95	79	83	76	180	190	171	1.28	1.30	1.25
		X-ray	12	107	112	102	54	54	54	161	166	156	1.98	2.07	1.89
3	980	Control	6	109	114	105	115	118	112	224	232	217	.95	.96	.94
		X-ray	12	115	115	114	98	96	101	213	211	215	1.16	1.20	1.13
4	980	Control	3	109	115	102	83	85	81	192	200	183	1.31	1.35	1.26
		X-ray	5	115	118	112	63	64	62	178	182	174	1.83	1.84	1.81

* Avg = Avg value for the 2 samples taken at 1.5- and 4-min. intervals after decapitation. 1.5 and 4 = values for the 2 samples taken at these intervals.

† Each value represents the avg of all animals in each group.

between the two sampling periods. DPNH was also determined at both intervals although it had been observed that this compound is relatively unchanged in normal liver samples taken at these periods(4). For the purpose of standardization, 2 samples from each control and X-rayed liver were tested at 1.5- and 4-minute intervals after decapitation. Prior to centrifugation of the boiled homogenates, aliquots were removed for the determination of total nitrogen content by the micro-Kjeldahl method.

Results. In Table I, the data for all post-irradiation periods studied are averaged together for the concentrations of DPN, DPNH, and DPN+DPNH based on the nitrogen content of the tissue; DPN/DPNH ratios are also presented. In Exp. 1 (700 r), 4 control and 4 X-rayed rats were sacrificed 5 hours after exposure and 2 control and 4 irradiated animals at 30, 73, and 150 hours following exposure. No significant change in the concentration of DPN was found at any of these periods, whether the level of liver coenzyme was expressed on a wet weight or nitrogen basis. The concentration of DPNH was likewise unchanged when related to nitrogen content but lowered by 13% when based on wet weight. In Exp. 2, the rats were given a total dose of 980 r. Two control and 4 irradiated animals were sacrificed at 15, 39, and 77 hours after X-raying. It can be seen that while the concentration of liver DPN remained unchanged at this dose of radiation, that of DPNH was diminished by an average of 32%. The decrease in DPNH concentration, which was apparent 15 hours after exposure and still evident at 77 hours, was the same whether the changes in optical density were related to the nitrogen content or wet weight of the liver samples assayed. In order to confirm the decline in DPNH concentration at 980 r, another group of animals (Exp. 3) was irradiated, and both X-rayed and control rats were studied at 15, 39, and 63 hours after exposure. DPNH levels were again somewhat lowered, but the average for the entire post-irradiation period was only –15 and –18% when based on nitrogen content and wet weight, respectively. In a 4th experiment involving 3 control and 5 X-rayed animals investigated at 15-21 hours after exposure to 980 r, the decrease in the concentration of liver DPNH amounted to 25%.

In no instance did irradiation appear to cause a significant change in the concentration of the oxidized coenzyme in liver samples taken 1.5 minutes after sacrifice. Since the usual decline in the DPN concentration of samples taken at 4 minutes was observed more consistently for control than for irradiated animals, DPN levels of the latter group at 4 minutes were consistently slightly higher than those of the controls. A larger series of animals would have to be studied before such a trend could be considered significant. The DPNH levels for the 1.5-and 4-minute intervals were essentially identical for the control rats as well as the exposed animals. The increased DPN/DPNH ratios and the decreased total DPN levels of the X-rayed livers (particularly Exp. 2 and 4) reflect the decreased concentrations of DPNH found at 980 r.

Discussion. On the basis of the results reported in this study, it may be inferred that the destruction of liver DPN, whether oxidized or reduced, is not a factor in the production of injurious effects following the exposure of rats to sub-lethal doses of ionizing radiations. Indeed, even at a lethal dose, the relatively small decrease in liver DPNH probably negates its involvement in the events leading to death from radiation sickness. It is of interest to compare the results described in this investigation with the *in vitro* findings of Barron *et al.*(2). They observed that a solution of DPNH became oxidized (decline in the absorption band at 340 mμ) after receiving 1000 r but that a solution of DPN was not reduced (no appearance of the 340-mμ band). Enzymatic reduction of the irradiated sample showed that the decrease in optical density at this wave-length was due to DPNH oxidation since the band at 340 mμ reappeared(2). On the other hand, the reduced product formed on irradiating oxidized DPN (45,000-50,000 r) in the presence of ethanol or lactate was tested for enzymatic activity with alcohol dehydrogenase and acetaldehyde and found to be rapidly oxidized, but only to the extent of 50% of the added nucleotide. The same events do not seem to occur under the *in vivo* conditions presented in this report. If the 15-32% decrease in the concentration of DPNH were due to oxidation to DPN, then the irradiated livers should have exhibited an equivalent rise in oxidized DPN levels. No evidence was found for such an increase in the amount of DPN; hence, it would appear that the lowering of the DPNH concentration is due to some change in the structure of the reduced nucleotide other than oxidation. Another possibility is that the absence of a 15-32% increase in the concentration of oxidized DPN reflects a combination of two effects, that is, partial destruction of oxidized DPN and the conversion of an equivalent amount of DPNH to DPN. It has been pointed out that in all 4 experiments a slight increase in the DPN concentration of irradiated livers was found.

In view of the results obtained in other investigations on the changes of biochemical components in tissues more radiosensitive than liver(3,5), studies are now in progress to determine the effect of varying levels of whole-body X-irradiation on spleen and thymus DPN and DPNH.

Summary. X-irradiation of intact rats with 700 r had little effect on the concentrations of oxidized and reduced DPN of liver, while a dose of 980 r lowered the level of reduced DPN by 15-32% without significantly altering the amount of oxidized coenzyme. Possible explanations for these results have been discussed.

We wish to thank Miss Jeanne Boyd for valuable technical assistance.

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Received February 11, 1955. P.S.E.B.M., 1955, v88.

Effect of Scurvy on Hexokinase Activity of Tissues of Guinea Pigs. (21604)

SACHCHIDANANDA BANERJEE AND PULAK KUMAR GHOSH.
From Department of Physiology, Presidency College, Calcutta, India.

Colowick and Kalckar(1,2) demonstrated that the enzyme hexokinase catalyses the transfer of the terminal phosphate group of adenosine triphosphate to glucose or fructose, yielding the corresponding hexose-6-phosphate and adenosine diphosphate. Price, Slein, Colowick and Cori(3) observed a decrease in the hexokinase activity of muscle and liver of alloxanised rats. Price, Cori and Colowick(4) also observed that the liver and muscle hexokinase were inhibited *in vitro* by certain fractions of anterior pituitary and adrenal cortical extracts and this inhibition was counteracted by insulin. This work was, however, not confirmed by Broh-Kahn and Mirsky(5) and Stadie and Haugaard(6). It was shown by Banerjee(7) that scorbutic guinea pigs showed degenerative changes in the islets of Langerhans. Banerjee and Ghosh (8) observed diminished insulin content of the pancreas in scorbutic guinea pigs. Banerjee and Deb(9-11) showed by indirect evidences that adrenal cortical activity was diminished in scorbutic guinea pigs. It was, therefore, of interest to study the hexokinase activity of the liver, muscle, pancreas and kidney of both normal and scorbutic guinea pigs.

Experimental. Male guinea pigs weighing between 240 and 320 g were fed a scorbutogenic diet(12) with a daily oral supplement of 5 mg ascorbic acid. Animals which grew well were selected and divided into 2 groups. Animals of one of the groups continued to receive 5 mg ascorbic acid per day while the second group of animals received no ascorbic acid supplement. All the animals received 2 drops of a concentrate of vit. A and D twice a week. The food consumption of the second group of animals was noted and the corresponding animals of the first group were pair fed to eliminate any effect of inanition.

On the 22nd day of the experiment both the scorbutic and the normal guinea pigs were killed after an over night fast by decapitation. Liver, kidney, pancreas and skeletal muscles were removed immediately, soaked in filter paper, weighed and homogenized in an ice cold all glass homogenizer with ice cold 0.12 M potassium phosphate buffer of pH 7.8 containing 0.15 M potassium fluoride in the proportion 5 ml buffer to 1 g tissue. 0.2 ml homogenate was taken in a 15 ml centrifuge tube, incubated at 30° with 0.3 ml of a medium consisting of glucose (0.0024 M), potassium ATP (0.005 M), magnesium chloride (0.005 M), potassium fluoride (0.05 M), potassium phosphate buffer of pH 7.8 (0.04 M), and potassium chloride (0.042 M), for 10 minutes. Control experiments were carried out using the complete medium as above but without ATP. After incubation 0.5 ml of 0.3 N barium hydroxide was added to arrest enzyme activity, 0.5 ml of a 5% zinc sulfate solution was added to precipitate proteins and to adsorb the glucose-6-phosphate, 3.5 ml water was then added, mixed and centrifuged. The centrifugate was filtered through Whatman No. 42 filter paper and glucose in the filtrate was estimated by the method of Hagedorn and Jensen(13). To estimate the glucose present in the homogenate, 0.3 ml water was taken in a centrifuge tube and the following were added in order, 0.5 ml barium hydroxide, 0.2 ml homogenate, 0.5 ml zinc sulfate solution and 3.5 ml water. Contents were mixed and centrifuged, and glucose was estimated in the filtered centrifugate. An aliquot of the homogenate was dried at 105° to constant weight to get the dry weight. The hexokinase activity was measured by the rate of glucose utilization according to the method described by Long (14) and was calculated in terms of micrograms of glucose disappearing per mg dry weight of tissue per hour (—Q glucose). Adenosine triphosphate used in the investigation was prepared from rabbit muscle as the dibarium salt according to the method of Dounce, Rothstein, Beyer, Meier and Freer (15). All the chemicals used were of analyti-

TABLE I. Hexokinase Activity of Tissues of Guinea Pigs (-Q Glucose).

	Liver	Skeletal muscle	Pancreas	Kidney
Normal (11)	67 ± 1.8*	85 ± 2.1	95 ± 2.0	76 ± 1.1
Scorbutic (11)	50 ± 2.1	78 ± 1.2	98 ± 2.5	92 ± 2.2
t	6.3	2.9	0.9	6.4

Figures in parentheses indicate No. of animals.
* Stand. error.

cal reagent grade. The results are given in Table I.

Discussion. A significant decrease in the hexokinase activity of the liver and skeletal muscle was observed in scorbutic guinea pigs. No change in the hexokinase activity was observed in the pancreas while the same in the kidney was considerably increased during scurvy. As the skeletal muscle and liver are mainly concerned in the utilization of glucose in the body, the diminished hexokinase activity in these 2 tissues in scurvy might be responsible for the altered carbohydrate metabolism observed by Banerjee and Ghosh (8). Insulin is said to counteract the anti-hexokinase activity of anterior pituitary and adrenal cortex. The diminished insulin formation in scorbutic guinea pigs(8), therefore, might be the reason for the decreased hexokinase activity in the muscle and liver.

Summary. Scurvy was produced in guinea pigs and the hexokinase activity of liver, skeletal muscle, pancreas and kidney was studied. The results were compared with the hexokinase activity of the same tissues of normal pair-fed controls. The hexokinase activity in the liver and skeletal muscle of scorbutic guinea pigs was significantly diminished. No change was noticed in the pancreas. Kidney from scorbutic guinea pigs showed enhanced hexokinase activity.

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Received February 18, 1955. P.S.E.B.M., 1955, v88.

Effect of Methionine and Casein on Acetoacetate Induced Hyperglycemia.
(21605)

M. C. NATH, C. H. CHAKRABARTI, AND S. G. NAYUDU.
From the University Department of Biochemistry, Nagpur, India.

Lazarow(1,2) reported that injection of large doses of glutathione immediately preceding a diabetogenic dose of alloxan completely protects rats from diabetes. Griffiths (3) showed that diabetes can be induced in rabbits by injection of uric acid only when the blood glutathione level is depleted by keeping the animals on a cystine-methionine deficient diet. He observed further that if the deficient diet was supplemented by 0.2% methionine the blood glutathione level did not fall appreciably nor did the rabbits develop hyperglycemia following uric acid injection. Nath and his associates(4,5) reported that gradual accumulation of the acetoacetate in the system is responsible not only for the onset of hyperglycemia but also for the development of other diabetic symptoms associated with decreased sugar tolerance in the experimental animals (rabbits) kept on Bengal gram (*Citer areatinum*) diet. This fat metabolite has further been shown in this laboratory to cause inactivation of endogenous insulin both *in vivo* as well as *in vitro* and to bring about considerable decrease in the potency of pancreatic insulin of guinea pigs on prolonged injection(6,7), also to bring about a marked depletion in blood GSH of these animals(8) and to increase their susceptibility to alloxan diabetes(9).

Tidwell *et al.*(10) have reported that they could not find any appreciable effect of sodium acetoacetate on glycogen storage in the liver and muscle of rats, neither could they observe any change in blood sugar except slight hypoglycemia(11). Since they used a different species of animals and a diet, in which the percentage of protein (casein) was higher than that of ours and since casein contains an appreciable amount of methionine, it was thought worthwhile to investigate the role of methionine and casein on acetoacetate induced hyperglycemia in rabbits with a view to account for the difference in behavior shown by the two different types of animals and those again kept on different diets.

Experimental. Forty healthy rabbits each weighing approximately 2 kg body weight were divided into 6 groups. The animals of Group I were kept on the cystine-methionine deficient diet prepared according to Griffiths(3). The animals of Groups II, III, IV, and V were fed cystine-methionine deficient diet supplemented with varying amounts of methionine as shown in Table I. The last group of animals were kept on cystine-methionine deficient diet in which 26% carbohydrate was replaced isocalorically by 26% casein. All the animals were kept in individual cages. Sodium acetoacetate was given intraperitoneally in increasing doses beginning from 50 mg/kg body weight. Blood sugar was determined periodically according to the method of Hagedorn and Jensen(12) and blood GSH was estimated by the method of Woodward and Fry(13). The results are shown in Tables I and II.

Results. Data as per Table I indicate that

TABLE I. Effect of Methionine and Casein on Acetoacetate Induced Hyperglycemia.

No. of rabbits	Nature of diet	Blood sugar, mg/100 cc, after dose of injection*					
		Initial	10	30	40	55	70
10	SH deficient	108.2 ± 4.5	114.7 ± 8.2	162 ± 10.2	172.9 ± 12.5	178.6 ± 10.5	183 ± 12.8
5	*Idem* + 0.2% methionine	108.8 ± 3.8	112.1 ± 4.8	160 ± 10.5	170 ± 8.5	173 ± 12.5	180.3 ± 15.5
5	" + 0.5% "	109 ± 4.8	115.8 ± 2.5	152.6 ± 12.8	170 ± 5.8	170.6 ± 5.2	179.6 ± 8.2
5	" + 1 % "	108.3 ± 4.2	109.6 ± 3.8	110.3 ± 4.2	112.6 ± 5.8	116 ± 4.0	124.3 ± 6.2
5	" + 2 % "	108.6 ± 3.8	110 ± 2.5	112 ± 2.2	110.8 ± 3.8	111 ± 3.2	112.4 ± 4.0
4	" + 26 % casein	108.3 ± 3.8	110.3 ± 3.2	111.2 ± 3.2	110.8 ± 3.8	112.8 ± 5.8	124.5 ± 7.5

* Daily dose of acetoacetate injection: 1st to 10th day, 50 mg/kg; 11th to 30th day, 100 mg/kg; 31st to 55th day, 150 mg/kg; 51st to 70th day, 225 mg/kg.

TABLE II. Effect of Methionine and Casein on the Blood GSH of the Animals (Rabbits) Injected with Acetoacetate.

No. of rabbits	Nature of diet	Blood GSH in mg/100 cc blood after days of injection					
		Initial	10	30	40	55	70
12	SH deficient	31.7 ± 1.9	25.1 ± 1.9	20.4 ± 1.4	18.6 ± 1.8	16.2 ± 1.6	14.5 ± 1.8
6	*Idem* + .2% methionine	32.4 ± 2.1	29.0 ± 2.6	21.5 ± 2.0	18.6 ± 2.4	16.3 ± 2.2	14.6 ± 2.2
6	" + .5% "	31.7 ± 2.5	29.3 ± 2.3	22.8 ± 1.6	20.5 ± 2.5	16.4 ± 2.2	15.5 ± 2.0
3	" + 1 % "	30.9 ± 1.2	30.3 ± 1.0	26.2 ± 1.3	25.0 ± 2.0	22.0 ± 2.2	20.8 ± 2.7
6	" + 2 % "	31.4 ± 1.7	30.5 ± 2.7	30.2 ± 2.2	29.9 ± 2.0	27.9 ± 1.7	27.1 ± 1.9
4	" + 26 % casein	31.7 ± 2.2	31.0 ± 1.9	29.8 ± 2.6	28.2 ± 2.1	24.3 ± 3.2	21.8 ± 2.4

methionine in large concentration leads to the prevention of hyperglycemia caused by daily repeated injection of acetoacetate. Methionine, which when given in lower concentration, *i.e.*, 0.2% to 0.5% of the diet, shows but slight effect on the prevention of hyperglycemia, is highly effective while used in higher concentration (*i.e.* 1%) up to a period of 40 days. If the concentration of methionine is increased still further (*i.e.* 2%) it can completely protect the animals from hyperglycemia throughout the period of experiment (*i.e.* 70 days) accompanied with only a slight fall in blood GSH. It can also be seen from Table I that animals maintained on the diet supplemented with casein (26%), as used by Tidwell *et al.*(11), show normal blood sugar values up to 40 days; but as the injection is continued for another 30 days, there is a slight tendency of hyperglycemia in these animals. Such hyperglycemia seems to be due to the insufficiency of methionine owing to the increased demand of such compounds, through the continuous injection of acetoacetate, as is evident from the fact that the animals supplemented with 2% methionine show no indication of the development of hyperglycemia at all.

The data further show that there is an inverse relationship between the blood sugar level and the glutathione concentration of blood. The greater the depletion of blood GSH, the greater is the value of blood sugar. Similar behavior has also been reported earlier from this laboratory(9). The animals, whose blood GSH concentration is maintained at the normal level by supplementing methionine in the diet, do not exhibit hyperglycemia thus showing that glutathione plays an important part in the prevention of acetoacetate induced hyperglycemia and the addition of the requisite amounts of methionine or casein protects the animals from the depletion of blood GSH.

Tidwell *et al.*(11) failed to observe hyperglycemia in rats kept on a diet containing 26% of casein through acetoacetate injection. We have also not observed any appreciable change in blood sugar of rabbits kept on such diet for 40 days. In a recent communication from this laboratory(14) it has been shown that rats require very high concentration of acetoacetate for producing hyperglycemia and glucosuria. In a previous communication, it was shown by Nath and Chakrabarti that β-hydroxybutyrate in lower concentration can deplete liver and muscle glycogen in rats on 20% casein diet(15). This difference in behavior of the two substances in rats was accounted for through the difference in their capacity for biosynthesis of ascorbic acid, which has been found to be stimulated in rats by sodium acetoacetate injection. But this biosynthesis of ascorbic acid was found to be retarded by acetoacetate in higher concentration. We observed hyperglycemia and depletion of glycogen storage in liver and muscle of rats by injecting sodium acetoacetate in higher concentration. The higher percentage of casein in the diet, which Tidwell *et al.* used in their experiments might account for their failure to notice hyperglycemia in rats by acetoacetate injection. Deficiency of SH compounds seems to be an essential factor for acetoacetate induced diabetes.

Further, it has recently been reported by Chari and Wertheimer(16), who have indicated clearly from their studies with rat diaphragm that acetoacetate at a concentration higher than that of glucose has a specific action on the retardation of glycogen synthesis even in presence of insulin; but while the concentration of acetoacetate is much lower than that of glucose (1:4) no such effect could be observed. This behavior of acetoacetate has also been confirmed in this laboratory(14). Details to be reported later.

Summary. 1. Hyperglycemia caused by daily repeated injection of acetoacetate to rabbits was completely prevented by supplementing 2% methionine to sulfhydryl deficient diet. 2. Methionine in lower concentration (0.2 to 0.5%), however, failed to prevent acetoacetate induced hyperglycemia. 3. A partial prevention of such hyperglycemia is observed when the concentration of methionine is maintained at 1% level. 4. Animals maintained on the diet supplemented with casein (26%) show normal blood sugar value up to 40 days. But as the injection is continued for another 30 days at the following doses of acetoacetate injection: 31st to 50th

day, 150 mg/kg; 51st to 70th day, 225 mg/kg, there is a slight tendency of hyperglycemia in these animals. 5. An inverse relationship between the blood glutathione concentration and blood sugar value has been observed. More the depletion of blood GSH, more is the rise of blood sugar brought about by the ketone bodies. 6. Deficiency of SH compounds seems to be an essential factor for acetoacetate induced diabetes in experimental animals.

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Received February 18, 1955. P.S.E.B.M., 1955, v88.

Attempts to Demonstrate Absorption of Hydrocortisone by New Chemical Test Following Inunction Into Human Skin.*† (21606)

Victor H. Witten, Alfred J. Shapiro, and Robert H. Silber.‡
(Introduced by M. B. Sulzberger.)

From Department of Dermatology and Syphilology, New York University Post-Graduate Medical School and Bellevue Hospital.

Since the first reports(1) it has been confirmed that topical applications of hydrocortisone are of value in treatment of certain previously intractable, resistant, or chronic dermatoses(2-7). Dermatologic use of hydrocortisone ointment is now one of the most favored topical therapeutic agents. While systemic administration of cortisone or hydrocortisone or of ACTH is more effective in many cases, absence of the serious, sometimes dangerous systemic effects, when hydrocortisone is used externally, make this an infinitely safer and therefore definitely preferable procedure. This lack of harmful systemic effects might be explained, theoretically, by assuming that when applied topically the hormone penetrates skin lesions sufficiently to be therapeutically helpful, but does not penetrate to deeper tissues or blood stream in sufficient quantity to exert possible harmful systemic effects. In many thousands of cases which have now been treated, prolonged topical use of hydrocortisone ointments over large skin areas has, as far as we know, not produced a single instance of clinically discernible systemic action. The relatively few attempts to demonstrate percutaneous absorption of hydrocortisone in ointments have so far failed. Such studies have utilized circulating eosinophile counts(8) as the index of hydrocortisone effect in subjects with normal and diseased skins(9).

In view of the increasing use of topical hydrocortisone preparations in many dermatoses

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* Aided by grant from Mrs. J. Hazen, M. L. Annenberg Foundation, and Merck & Co., Rahway, N. J.

† We wish to thank Dr. Dorsey C. Gamsjager, Bellevue Hospital, for his assistance.

‡ Merck Institute for Therapeutic Research, Rahway, N. J.

of infants, children and adults and because of its possible continued use in ever greater quantities and in increasing numbers of patients with involvement of larger body surfaces, we considered it important that investigations on possible absorption be continued and enlarged. Introduction of a new and more sensitive test to measure systemic absorption of hydrocortisone(10) seemed to lend itself ideally to more exact exploration of this problem. The colorimetric test used detects the presence of 17, 21-dihydroxy-20-ketosteroids. This includes hydrocortisone, and in addition certain of its metabolic derivatives. The unconjugated form which is measured in plasma has been shown to be almost exclusively hydrocortisone(11). In the urine, hydrocortisone and its metabolites are found.

TABLE I.

Urinary excretion of 17,21-dihydroxy-20-ketosteroids (expressed in mg/g creatinine)			Blood values of 17, 21-dihydroxy-20-ketosteroids (expressed in γ %)	
Specimen			Specimen	
Pre-I*	Post-I	Post-oral hydrocortisone	Pre-I	Post-I
Normal subjects				
4.8	3.4	12.2	17	9
5.3	5.1	25.8	9	5
6.2	6.9	18.4	25	6
2.0	2.6	11.0	26	19
3.1	2.9	16.5	26	17
3.3	3.8	17.6	Insufficient sample	17
Patients with skin disease				
2.8	3.5	9.6	30	11
2.2	3.5	21.6	†	†
‡	‡	‡	‡	‡
2.7	4.6	17.0	19	22
2.8	3.0	20.0	13	22
3.3	4.4	18.5	16	15
6.2	5.7	25.6	13	17
21.5	11.0	10.5	27	22
4.3	4.3	24.0	26	23

* I = Inunction.

† Lost.

‡ Interfering substance.

Method. Six normal adult male volunteers and 9 adult patients (3 ♀ and 6 ♂) with extensive or generalized skin disease (bullous erythema multiforme, allergic eczematous contact-type dermatitis, pemphigus foliaceus and psoriasis) were used. Each of 15 subjects was to have a given amount of hydrocortisone ointment rubbed into large areas of skin, and urine and blood subsequently analysed for presence of 17, 21-dihydroxy-20-ketosteroids. These blood and urine levels were compared with the levels in specimens taken before application of the hormone and also with levels produced by later oral ingestion of hydrocortisone. Each of the specified steps was carried out at the same time in volunteers and patients. The first specimen of urine was collected during 12 hour period prior to inunction of hydrocortisone ointment. A specimen of venous blood was drawn just prior to application of ointment. These will be referred to as *pre-inunction* or baseline urine and blood specimens. Following collection of these specimens, each subject was rubbed with hydrocortisone acetate 2½% ointment in a washable ointment base (consisting of zinc stearate, propylene glycol, polyethylene glycols, and water). An area of the body usually including back, shoulders and chest and both palms was rubbed well for 10 minutes at each inunction. Inunctions were done 3 times daily for 3 consecutive days. Approximately 3.3 g of ointment containing about 82 mg of hydrocortisone acetate was applied with each rubbing. Hence, a total dosage of 750 mg of hydrocortisone acetate was applied over the 3 day period. Immediately following the 3 day inunction period, another 12 hour specimen of urine was collected and a second blood specimen drawn. These will be referred to as *post-inunction* urine and blood specimens. After collection of post-inunction urine and blood specimen each subject was given 40 mg of hydrocortisone free alcohol by mouth following which a third 12 hour urine specimen was collected. This will be referred to as *post-oral hydrocortisone* urine. Subjects did not bathe during the experiment. Special precautions were taken to avoid contamination of urine specimens with the ointment by parts of the body adjacent to and including the penis. Female patients were catheterized.

The *results* are tabulated in Table I. Presence of an aldehyde in urine and blood from paraldehyde which had been taken by mouth interfered with the determination of 17, 21-dihydroxy-20-ketosteroids in case #9.

The high urinary excretion values for 17, 21-dihydroxy-20-ketosteroids in pre- and post-inunction specimens for case #14 suggests some previously existing hormonal abnormality; this patient had not been on steroid therapy. In the remaining 13 subjects in Table I there is *no significant alteration* in pre-inunction and post-inunction urinary 17, 21-dihydroxy-20-ketosteroid levels. Table I again shows *no significant alteration* in pre-inunction and post-inunction blood 17, 21-dihydroxy-20-ketosteroid values. We are unable to account for the unusually low post-inunction blood levels in Cases 1, 2, 3, and 7.

In contrast to these findings, oral administration of a single dose of 40 mg of hydrocortisone was sufficient to produce a significant increase in urinary 17, 21-dihydroxy-20-ketosteroids in all 13 subjects (Table I).

Conclusions. Inunction of relatively large body areas of normal or diseased skin with 30 g of ointment containing 750 mg of hydrocortisone acetate over a 3-day period does not increase the 17, 21-dihydroxy-20-ketosteroid levels in urine and blood. This study does not rule out the possibility that hydrocortisone is converted to still another compound or so bound with other molecules as not to be detectable by the method used, even though absorption has taken place. The findings lend further support to the mass of clinical evidence indicating that there are no dangers to be anticipated from absorption and consequent systemic effects of therapeutic quantities of hydrocortisone applied topically in ointment form even to large areas of altered skin for long periods of time.

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Received January 11, 1955. P.S.E.B.M., 1955, v88.

Effect of Massive Antigen Dosage on Antigen Retention and Antibody Response in Rabbits.* (21607)

ARTHUR G. JOHNSON,[†] DENNIS W. WATSON, AND WILLIAM J. CROMARTIE.[‡]

From Department of Bacteriology and Immunology, University of Minnesota, Minneapolis.

The mechanisms by which the host eliminates antigens may bear a relationship to serum sickness and the development of late sequelae in streptococcal infections(1). It is well known that certain individuals when exposed to the etiological factors of these diseases may remain free from the symptomatology while others will show the characteristic pathological entity. It is quite possible that the rate of elimination of antigenic materials may be responsible for this variation in response by individuals. Of the present known mechanisms concerned in the elimination of an antigen by a host, the production of

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[*] This investigation supported by research grant from the National Heart Institute, of the National Institutes of Health, Public Health Service.

[†] Present address: Army Medical Service Graduate School, Walter Reed Army Medical Center, Washington, D.C.

[‡] Present address: Medical School, University of North Carolina, Chapel Hill.

antibodies, and resulting capacity to immobilize or neutralize and eliminate the antigen by subsequent phagocytic action has been implicated as the main functional unit. Talmage, *et al.*(2) have interpreted the rate of protein antigen catabolism as an indicator of the degree of antibody response. However, injections of relatively large doses of one type of antigen, pneumococcal polysaccharide, were observed by Felton(3) to so alter the immunological system that antibody appeared to be no longer produced by a subsequent immunizing dose. This phenomenon, designated "immunological paralysis," was found to be type specific(3,4). The isolation of pneumococcal polysaccharide from tissues of human beings who had succumbed to diseases other than pneumococcal pneumonia suggests that retention of this antigen may result in a hypersusceptible state in man. The accumulation of this polysaccharide in tissues of the host over a period of years could conceivably result in vulnerability of the host by suppression of specific antibody formation. The alteration of the defense mechanism by large doses of an antigen was shown by Metchnikoff as early as 1883(5) when he noticed the inhibition of phagocytosis by high concentrations of yeast spores. Similar findings have been reported by others(6,7). Recently, Dixon and Maurer(8) have reported briefly on the effects of repeated massive doses of antigen in rabbits.

This study was based on the assumption that host response to massive quantities of antigen would facilitate interpretation of the mechanism of elimination of antigen and of antibody formation. This report records the effects of excessive "saturation" doses of a relatively pure antigen, bovine albumin, on the immune response of rabbits. The rate of elimination was measured by quantitative precipitin determination of the serum bovine albumin level.

Materials and methods. Antigen. A 10% solution of bovine albumin, fraction V (Armour and Co.), in physiological saline was adjusted to pH 7.0. Merthiolate was added to a final concentration of 1:10,000, sterility achieved by Seitz filtration, and the solution kept in storage at 4°C. Appropriate dilutions were used for the various tests. *Antiserum* was prepared by injecting rabbits intravenously and/or subcutaneously 4 times a week for 4 weeks with 2 mg of the above antigen. Ten days after the last injection, blood was withdrawn by cardiac puncture and the serum separated. Those animals having a high titer of anti-bovine albumin were bled again and all sera pooled. Complement was removed by the addition of optimal concentrations of the horse serum and rabbit anti-horse serum system. The serum was merthiolated, sterilized and stored as was the antigen. *Quantitative precipitin determinations.* The quantitative precipitin technic for determination of either antigen or antibody was essentially that of Kabat and Mayer(9). Blood was obtained from the marginal ear vein or by cardiac puncture; the serum was stored at $-56°C$ until sufficient samples were collected for assay. All samples were recentrifuged before testing. In order to analyze serum samples for antigen, a calibration curve was first made on the basis of the addition of increasing amounts of antigen to a constant amount, 1 ml, of antiserum. Saline was added to a total volume of 3 ml. The micrograms of bovine albumin nitrogen in the unknown antigen samples were determined by interpolation on the calibration curve after determination of the total nitrogen precipitated by the micro-Kjeldahl technic. Each analysis was carried out in duplicate and tests on all supernatant fluids showed that the dilution of serum reacted with antibody in the region of antibody excess. Appropriate antiserum and antigen controls were run throughout and modifications for samples of 10-100 μg total nitrogen were made.

Results. I. Rate of elimination of massive doses of bovine albumin. Eleven normal New Zealand white rabbits of approximately the same weight (2.5 kg) were injected intraperitoneally with one gram bovine albumin solution per day for 5 days. Although the individual blood clearance rates of antigen were found to vary following quantitative analyses, the response of the rabbits appeared to fall into 2 relative groups: (1) 5 rabbits which retained residual antigen and (2) 6 rabbits which appeared to have cleared the antigen

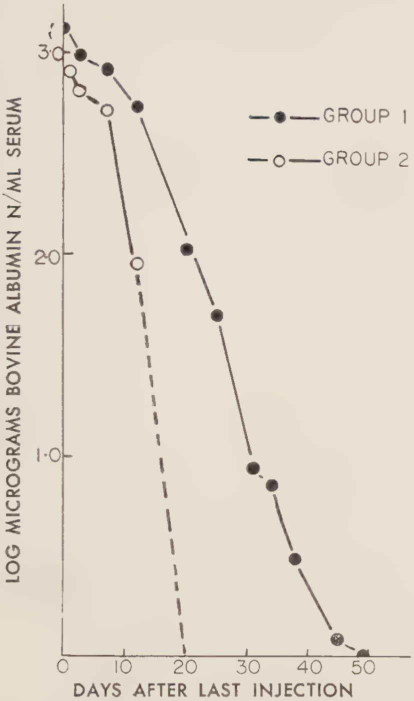

FIG. 1. Geometric mean elimination rates of bovine albumin antigen by New Zealand rabbits.

completely from the circulation (Fig. 1). Four of the rabbits comprising the latter group were found to possess anti-albumin after antigen had disappeared from the serum with only one retaining serum antigen as long as 20 days. Of the 5 animals which retained the antigen, only 1 showed demonstrable serum antibody after antigen elimination. As no antibody was detectable in the serum of the other 4 up to 108 days after the last injection, it is reasonable to assume that residual but undetectable antigen was still present within the host, suppressing antibody formation. Six months after the initial injection, one of the latter animals responded with specific antibody to the subcutaneous injection of 20 mg bovine albumin. The rate of elimination of the antigen for each rabbit occurred in 3 phases: (1) an initial rapid phase probably manifesting early dilution, (2) a plateau of early antigen retention for several days, and (3) final clearance. The third phase varied for each animal and allowed segregation into the 2 groups described.

Statistical analysis of the data supports the grouping of the rabbits studied. By the method of components of variance, 91% of the source of the variation in the rabbits comprising Group 1 may be attributed to time lapsed between analysis, only 3% to inherent differences in the individual rabbits, and 6% as a result of all other factors. For the curve representing those rabbits which have shown the ability to rid their blood streams of detectable antigen, 60% of the variation occurs as a result of time, 20% to individual rabbit variation, and 20% to all other causes. The coefficient of variation, illustrating the per cent variation of the members of each group on any one day is approximately 15% for the *curve* of the above group in contrast to a coefficient of variation value of approximately 50% for those rabbits showing early antigen clearance. The results of the 2 statistical methods make it evident that the antigen-retaining rabbits are much more homogeneous as a group than Group 2. Presumably the lack of homogeneity and variable time course of elimination in the latter reflects a clearing mechanism (which is inherently variable) functioning in these animals.

II. Effect of various conditions on rate of elimination of massive doses of bovine albumin in American Dutch rabbits. From the preceding experiment the impression was gained that antibody formation might be suppressed until all antigen had been eliminated from an antibody-forming site. Accordingly further evidence for this assumption was sought in the following three experiments. *A. Normal animal*: A female rabbit was given intraperitoneally 1 g bovine albumin daily for 10 days. On the ninth day, 0.5 mg antigen was injected intracutaneously preceding the scheduled injection for the purpose of detecting antibody to the albumin. No evidence of an Arthus reaction was observed. Twenty-four days following cessation of antigen injections, a second 5 day series of similar injections was administered to determine whether there was any difference in the rate of disappearance from the circulation following the previous "saturation" dosage and subsequent rest. The serum level of antigen was followed quantitatively for a period of 11 weeks with the results illustrated in Fig. 2. It may be

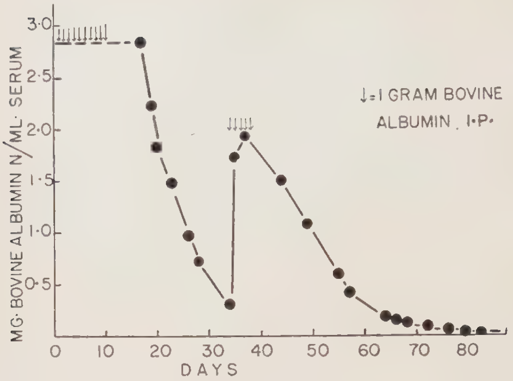

FIG. 2. Rate of elimination of massive doses of bovine albumin under varying conditions by a normal American Dutch rabbit.

seen that the initial rate of elimination following the second course of injections is no greater than that observed following the first series. As the number of days increases, a very slow rate of albumin elimination is seen, with residual antigen quantitatively detectable in the serum for approximately 8 weeks after the last injection. *Qualitative analysis* before the second series of injections demonstrated the presence of antibody coexisting with antigen. However, this antibody was not believed to be directed towards bovine albumin. Previous work in this laboratory(10) has shown crystalline bovine albumin to be heterogeneous immunologically. Furthermore, Cohn and associates (12) in their work with rabbits immunized with purified conalbumin found more antibody produced against a minor component present in the preparation than against the conalbumin itself. Accordingly, a qualitative test for precipitins to a trace of bovine globulin believed to be present in the antigen used in this investigation, was carried out on several of the serum samples showing simultaneous occurrence of antigen and antibody. Using bovine globulin as antigen, a precipitate resulted in each instance, while controls were negative. This would indicate that the antibody demonstrated in the apparent presence of antigen was in reality formed to a globulin impurity and not the bovine albumin *per se*.

When neither antigen nor antibody could be demonstrated in the serum for a period of 16 days, the rabbit was sacrificed. Post mortem findings were negative. The absence of any enlargement or abnormality in the lymph nodes was of interest. Microscopic examination of sections of the liver and kidney showed no pathological changes.

B. Immunized rabbit. The following experiment was undertaken to observe the effect of antibody to bovine albumin on the rate of elimination of massive doses of the homologous antigen. After a titer of 0.25 mg antibovine albumin nitrogen per ml had been developed in a male rabbit, 0.63 g bovine albumin was given intraperitoneally in small amounts during an 8 hour period. This was followed with a total of 6 grams given over a period of 7 days. Serum samples obtained at intervals were analyzed for bovine albumin. The results illustrated in Fig. 3 show that the retention of residual antigen in the serum (phase 3) did not occur since the antigen concentration rapidly falls off to zero.

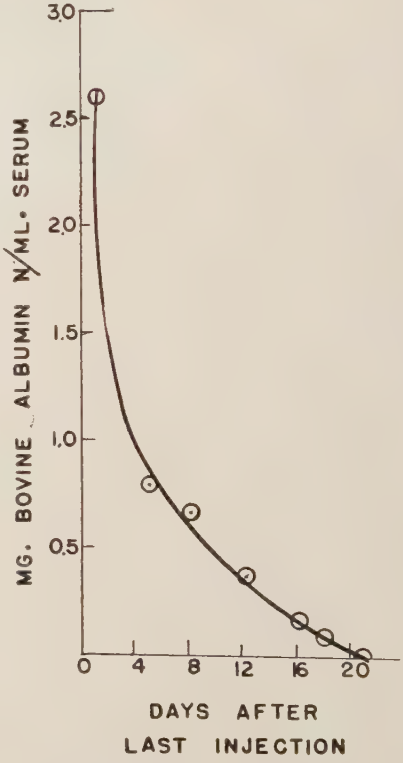

FIG. 3. Rate of bovine albumin elimination by an immunized American Dutch rabbit.

Antibody subsequently appeared in the serum.

C. Refractory rabbit. In this experiment the rate of elimination of large doses of bovine albumin was studied in a rabbit previously showing a poor antibody response to immunizing injections of this antigen. A male rabbit that had previously produced only a trace of precipitins when subjected to the immunization schedule described, was used. Three "booster" doses of 5 mg bovine albumin given intravenously and spaced at intervals of 12, 50 and 40 days from the previous injection failed to produce precipitins. Seven weeks following the last of these injections, this rabbit received a total of 6.6 g bovine albumin intraperitoneally over a period of 8 days. On the forty-fourth day after the last injection, when the bovine albumin had reached a concentration of 0.02 mg per ml, an attempt was made to neutralize completely, by passively transferred antibody, all tissue and serum antigen within the animal, thereby permitting the detection of "free" uncombined antibody. Accordingly, 37 ml of antibovine albumin rabbit serum of high titer was slowly injected; an additional 50 ml was given on the 52nd day. Twenty days later when no antigen was demonstrable in the serum, an additional 20 ml of antiserum was administered. Antibody was then demonstrable over a period of 19 days with no bovine albumin detectable in the serum. Since quantitative analysis revealed that the antibody level did not rise but decreased steadily, this response can probably be attributed to the antibody which was passively transferred.

To determine now the mode of elimination of antigen by this animal, an exactly similar series of injections was repeated. Fig. 4 compares the elimination rates under the two conditions, and shows their parallelism. On the 98th day following the last injection of the second series, a skin test for detection of antibody elicited no response. At no time throughout the course of the experiment was antibody detectable in the presence of antigen.

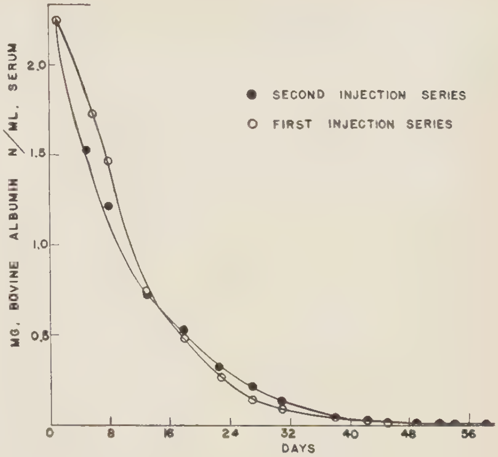

FIG. 4. Rate of elimination of bovine albumin by a refractory American Dutch rabbit.

Discussion. The oft-noted variability of individual host response to antigenic stimulus is substantiated by the data. Those rabbits which possess a mechanism capable of eliminating antigen relatively quickly have shown the ability to respond with antibody. However, it is interesting to note the apparent inability of antibody to bovine albumin to appear in a relatively high percentage of rabbits for a considerable length of time after all detectable antigen has been removed. An explanation for this may lie in the assumption that high antigen concentrations completely saturate all antibody-forming sites in the tissues and organs, resulting in an inability to synthesize antibody. These findings corroborate those reported by Felton(3) who suppressed immune mechanisms in mice with large doses of polysaccharide. The possibility exists that antibody is being synthesized but is immediately held in combination with the antigen. Three points in the experimental data presented here suggest that this does not occur. First, if antibody had been synthesized and was acting as a functional unit in ridding the antigen from these animals, it would be expected that the retention of antigen shown would not exist. As the concentration of antigen diminishes, more antibody should become available to neutralize the remaining antigen. The final retention portion of the curves should then drop at a rate much faster than that actually shown in phase 3. This is verified by the curve showing the clearance rate of massive doses of bovine albumin by an animal with circulating precipitins. One would also expect a faster rate of antigen removal after secondary exposure. This did not occur in Experiment IIA or IIC. Finally, the negative findings for any lesions suggesting the hypersensitive state may signify the absence of tissue-held antibody. Lesions attributable to the hypersensitiveness of rabbits to bovine albumin have been described(11,13).

The results obtained in the initial experiment confirm the substantial data of Talmage *et al.* in showing that the rate of elimination of antigen is an indicator of the degree of immune response. However, the rapid clearance of bovine gamma globulin from rabbit blood found by these investigators is attributed by them to antibody. While it is unquestionable that antibody, when present, markedly accelerates antigen removal, the formation in the presence of antigen of an immune response synonymous with antibody is difficult to reconcile with the evidence presented here. With the technic of massive doses of antigen, bovine albumin elimination curves show retention over a prolonged period of time when antibody, if synthesized, should be present in sufficient quantity to remove all residual antigen rapidly. In the authors' experience it has been the rare animal that does not respond with antibody to bovine albumin. It is possible, therefore, that the antigen retention shown in this study is not the result of lack of ability to form antibody, but rather that "poor antibody formers" as such, are a result of inability to rid antigen from the tissues.

The coexistence of antigen and antibody after intravenous injection of serum has been demonstrated repeatedly(13,14). It is possible that this phenomenon may be attributed to the faster rate of clearance of those antigenic components present in minute amounts. Antibodies to these would then be detectable, while the main component was still present at the antibody-forming site suppressing the reaction forming its antibody.

The mechanisms involved in serum sickness and the late sequelae associated with streptococcal infections may depend on the rate of elimination of antigen by the various organs of the host. Ancillary evidence for this hypothesis may be found in a study by Kendall (15) of two patients receiving 25 g of crystalline bovine albumin, one of whom developed serum sickness. Quantitative study of the rate of elimination of this antigen from the circulation of one patient showed antigen remaining detectable for as long as 140 days. With the retention of antigen and apparent absence of antibody, serum sickness did not develop. In contrast, the other exhibited a much faster antigenic clearance rate, terminating after 30 days with subsequent serum sickness. A hypothetical mechanism functioning might involve antigen being retained in the heart while it has been eliminated in the spleen. Antibodies are then formed in the splenic tissue, carried by the circulation to the heart wherein the antigen is attached to the cells, with the interaction resulting in the characteristic lesions and reactions.

Summary. Antigen elimination rates for massive doses of bovine albumin from the circulation of rabbits were resolved into two relative groups: (a) those eliminating the antigen rapidly, and (b) those retaining residual antigen for an extended period. All antibody-forming sites were assumed to be saturated with this antigen, and in the latter group antibody formation was suppressed. Data are presented suggesting that the breakdown and/or removal of an antigen from an antibody-forming site may be a necessary prerequisite to antibody formation. The rates of elimination of antigen from the various organs of the host are discussed in relation to postulated mechanisms concerned with serum sickness and the late sequelae of streptococcal infections.

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Received January 16, 1955. P.S.E.B.M., 1955, v88.

Uric Acid Metabolism in Hepatolenticular Degeneration.[*] (21608)

J. P. Mahoney,[†] A. A. Sandberg,[‡] C. J. Gubler, G. E. Cartwright, and M. M. Wintrobe.

From Department of Medicine, University of Utah College of Medicine, Salt Lake City.

Bishop, Zimdahl, and Talbott(1) have reported low serum urate levels in a brother and sister with Wilson's disease. Using N^{15} labeled uric acid these authors demonstrated that the uric acid pool in their patients was reduced and that the turnover rate of uric acid was about twice normal. In one of the patients 98% of the uric acid turned over was excreted by way of the kidney. To explain these abnormalities they suggested that there is in Wilson's disease a renal defect which results in inhibition of tubular reabsorption of filtered uric acid. The tendency of copper to form stable complexes with nitrogen-containing compounds such as amino acids is well known(2). The precipitation of copper-purine complexes has been described as a method for isolating purine compounds, including uric acid(3). Since the concentrations of direct-reacting serum copper in patients with Wilson's disease are abnormally high and since large amounts of copper are excreted in the urine(4), we considered the possibility that the abnormalities of uric acid metabolism in this disease might be the result of the formation of a uric acid-copper complex which cannot be reabsorbed by the normal kidney tubule.

The purpose of this paper is to present the results of studies on the serum uric acid level and the urinary excretion of uric acid in patients with Wilson's disease. In addition, the validity of the above two theories has been tested by comparing the ability of patients and normal subjects to excrete injected uric acid and by correlating the excretion of uric acid and copper by normal subjects and patients with Wilson's disease.

Methods. Uric acid was measured by the differential spectrophotometric method of Kalckar(5) as modified by Praetorius(6). Uricase was prepared by the method of Muller and Bauer(7). The methods for the determination of total plasma copper(8), the direct-reacting fraction of plasma copper(9), and urine copper(10) have been described. Creatinine was determined by the method of Bonsnes and Taussky(11). For *intravenous infusion,* a solution of uric acid was prepared by dissolving the appropriate amount in 0.8%

TABLE I. Serum Uric Acid Values (mg/100 ml) in Normal Subjects and in Patients with Wilson's Disease.

Group		No.	Mean	Range
Normal	♂	12	6.0	4.5-8.3
	♀	11	4.7	3.4-6.0
Wilson's disease	♂	11	1.8	.8-4.2
	♀	5	1.8	.8-2.9

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[*] This investigation was supported by a research grant from the National Cancer Institute, National Institutes of Health, Public Health Service.

[†] Public Health Service Research Fellow, National Heart Institute.

[‡] Scholar in Cancer Research, American Cancer Society.

TABLE II. Urine Uric Acid, Creatinine and Uric Acid/Creatinine Ratio in 5 Patients with Wilson's Disease.

	Body wt, lb	Uric acid mg/24 hr	Creatinine, mg/24 hr	Uric acid/ creatinine ratio
Normal range		276-460	1000-1800	.22-.43
Del. H.	70	620	753	.82
J. S.	100	462	856	.54
D. C.	165	542	1024	.53
Dav. H.	65	385	429	.90
B. S.	165	743	1310	.55
Mean	113	550	874	.67

aqueous lithium carbonate. This was passed through a Seitz filter, transferred to an infusion bottle and diluted to 500 ml with a 5% glucose solution. *Purity of the uric acid* used was checked by the determination of both the nitrogen content and the extinction coefficient in the ultra-violet range after the addition of uricase(6). *All* the *patients* with Wilson's disease, in addition to other evidences of the disease, had Kayser-Fleischer corneal rings. The total serum copper level of 12 of the 16 patients was less than 68 μg/100 ml. The serum copper levels of the other 4 patients were 72, 77, 90 and 100 μg/100 ml, respectively. The daily urinary excretion of copper was between 100 and 500 μg in all 10 of the patients on whom this determination was performed. The abnormalities in copper metabolism in this group of patients will be described more fully in a separate publication (12).

Results. The mean and range of values for serum uric acid in 23 normal subjects and in the 16 patients with Wilson's disease are given in Table I.

Concentration of uric acid in the serum of each patient with Wilson's disease was less than the lower limit of normal for the sex. In only one of the 16 patients was the value greater than 2.9 mg/100 ml. This patient (A.S.) is asymptomatic and has no neurologic manifestations(12).

The excretion of uric acid in the urine of 5 of the patients is recorded in Table II. Because of the marked differences in body weight the values are compared with the amount of creatinine excreted. It will be noted that all of the patients excreted increased amounts of uric acid per mg of creatinine excreted.

To explore the possible causes of these abnormalities of uric acid metabolism, the following experiment was carried out in a patient and in a normal subject of each sex.

During a baseline control period of 3 days, serum uric acid was measured twice daily and the urinary excretion of uric acid was determined over 12 hour periods. Following this baseline period, uric acid was infused intravenously during a period of 70 minutes. Each of the 2 normal subjects received one gram of uric acid. The 2 patients with Wilson's disease were each given 0.8 g. Serum levels of uric acid and copper were determined immediately before and at the termination of the infusion and, thereafter, at 4 hour intervals until baseline levels were reestablished. The renal excretion of uric acid and copper was determined over 4 hour periods. Throughout the experiment all subjects were maintained on diets low in purines.

The results of this study are presented in Table III and Fig. 1 and 2. As shown in Table III the hyperuricemia and hyperuricosuria induced by the infusion of uric acid were not accompanied by an increase in the serum copper level or by an increase in the amount of copper excreted in the urine. The coefficient of correlation between the urinary copper and uric acid in the two patients was + 0.25.

The rate of decline of the serum uric acid

TABLE III. Serum and Urine Uric Acid and Copper Values before and after the Intravenous Infusion of Uric Acid.

		Normal subjects		Wilson's disease	
		J. M.	M. O.	J. S.	D. C.
Serum uric acid, mg/ 100 ml	Before	5.2	2.5	1.0	1.7
	After*	12.2	9.1	7.5	5.4
Serum copper, μg/ 100 ml	Before	105	86	39	45
	After*	100	90	35	30
Urine uric acid mg/ 24 hr	Before	445	430	462	542
	After	892	906	1226	1252
Urine, copper, μg/ 24 hr	Before	14	6	354	264
	After	7	0	291	148

* At time of termination of infusion.

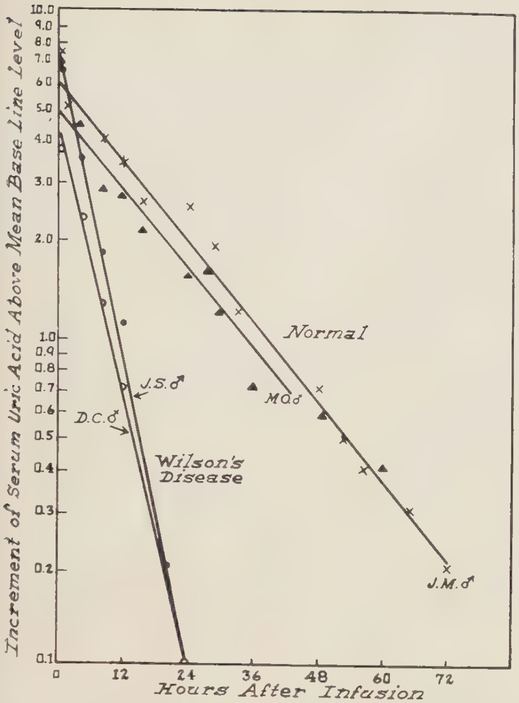

FIG. 1. Increment of serum uric acid above mean baseline level in 2 normal subjects and in 2 patients with Wilson's disease after intravenous administration of uric acid.

level following the intravenous infusion of uric acid is depicted in Fig. 1. The serum uric acid concentration of the patients returned to the pre-infusion levels in 24 hours, whereas, in the normal subjects, more than 72 hours were required.

The cumulative excretion of the uric acid in the urine above the mean base-line value is shown in Fig. 2. The patients with Wilson's disease excreted 90% of the injected dose during the first 24 hours. The normal subjects excreted only about 45% of the injected dose in this period.

Discussion. The studies reported herein demonstrate that in patients with Wilson's disease there are several alterations in the metabolism of uric acid; namely, (1) a low level of uric acid in the serum, (2) an increased excretion of uric acid in the urine, and (3) following the intravenous injection of a large amount of uric acid, a more rapid excretion of uric acid in the urine than occurs in normal subjects. Since it has been shown (13) that in normal subjects about 90% of the uric acid filtered through the glomeruli is reabsorbed by the tubules and that the glomerular filtration rate is either normal or decreased in Wilson's disease(14), the alterations in uric acid metabolism can be most easily explained on the basis of a decreased tubular reabsorption rate of uric acid in such patients.

These results are in agreement with those of Bishop, Zimdahl, and Talbott(1).

The aminoaciduria which occurs in Wilson's disease has been interpreted as evidence for a renal defect in this disease(15,16). Likewise, glycosuria with a normal serum glucose level has been observed in some patients who have this disease(17). The observations on uric acid offer additional evidence of a renal defect in Wilson's disease.

It is interesting that hypouricemia was present in all of the 16 patients studied. Two of the patients with low levels of serum uric acid (2.4 and 2.8 mg/100 ml) have not manifested neurologic abnormalities and do not excrete increased amounts of aminoacids in the urine. In a third patient[§] without neurologic disease and without significant symptomatology the serum uric acid level (4.2 mg/

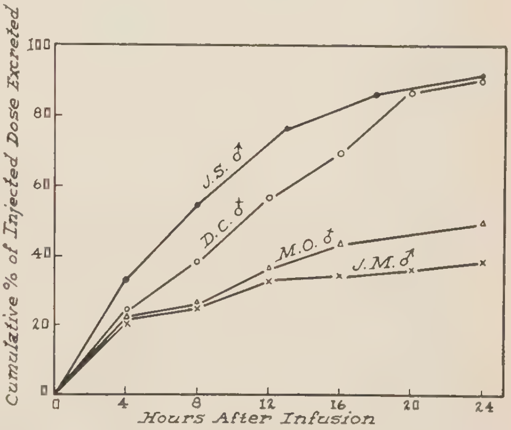

FIG. 2. Urinary excretion of uric acid following intravenous administration of uric acid to 2 normal subjects (M.O., J.M.) and to 2 patients (J.S., D.C.) with Wilson's disease.

Values are expressed as the cumulative per cent of injected dose excreted above the mean baseline values.

[§] We are indebted to Dr. J. G. Palmer, University of N. Carolina, for supplying serum from this patient.

100 ml) was only slightly below the lower limit of normal. Unfortunately, the quantity of alpha amino nitrogen in the urine of this patient was not determined. These observations suggest that the alterations in uric acid metabolism occur early in the course of the disease.

Since no increase in serum copper or in the amount of copper excreted in the urine accompanied the increase in uric acid in the serum and urine following the administration of a large dose of uric acid, it is improbable that chelation of copper with uric acid occurs in such patients to any appreciable degree.

Summary. It has been found that in patients with hepatolenticular degeneration (Wilson's disease) the level of uric acid in the serum is reduced and the amount of uric acid excreted in the urine is increased. Following the intravenous administration of uric acid, such patients excrete uric acid in the urine more rapidly than do normal subjects. No correlation was observed between the excretion of copper and that of uric acid. It is suggested that the alterations in uric acid metabolism are due to an inability of the renal tubules to reabsorb uric acid efficiently.

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Received February 7, 1955. P.S.E.B.M., 1955, v88.

Epidemic Influenza B and C in Navy Recruits, 1953-1954. I. Antigenic Studies on Influenza Virus, Type B.* (21609)

R. L. WOOLRIDGE, J. L. DEMEIO, J. E. WHITESIDE, AND J. R. SEAL.
(Introduced by Clayton G. Loosli.)

Of the Naval Medical Research Unit No. 4, U. S. Naval Training Center, Great Lakes, Ill.

There have been continual observations made on influenza and other acute respiratory diseases in recruits at this Naval Station since 1948. During epidemic influenza outbreaks of 1949, 1950, and 1951, A prime viruses were isolated(1). An epidemic of influenza B and C was reported in Navy recruits during the winter of 1952 by this laboratory(2). At this time 14 influenza C and 11 influenza B viruses were isolated. During an epidemic of influenza A prime in 1953 however, no influenza B or C virus isolations or serological rises were obtained(3). During the past winter only one serological rise to influenza A prime was found, whereas influenza B and C were prevalent as indicated by the following studies. The present report contains studies on 37 strains of influenza virus, type B, which were isolated between March 12 and April 16, 1954(4).

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* From Research Project, NM 005 051.06, Bureau of Medicine and Surgery, Navy Department, Washington, D.C. The views and opinions expressed herein are those of the authors and cannot be construed as reflecting the views of the Navy Department or of the naval service at large. This was also part of continuing studies on influenza made in cooperation with The Commission on Influenza, Armed Forces Epidemiological Board, and the Influenza Information Center, World Health Organization.

Materials and methods. Nasal washings were routinely obtained from recruits with febrile acute respiratory infections as they reported at the regimental infirmary between Dec. 1, 1953 and April 17, 1954. Nasal washings were also obtained from men seeking treatment for acute respiratory infections without fever, and for complaints other than respiratory infections between Jan. 25 and Apr. 17, 1954. Blood specimens were obtained at the time of nasal washing and again 3 weeks later. The sera were frozen and stored at –24°C until used. The nose was washed with 20 ml of nutrient broth which was expectorated into a beaker containing 2 ml of horse serum. The washings were homogenized for 2 minutes in a Waring blender and then inoculated intra-amniotically into 13-day-old embryonated eggs. Prior to inoculation, 1000 units of penicillin and 1 mg of streptomycin were added per ml of homogenized nasal washing. After incubation for 3 to 4 days at 35°C the amniotic fluid was harvested and tested with 0.5% suspension of chicken and guinea pig erythrocytes. Tests were read after 60 minutes at 4°C. At least 2 egg passages were made before a specimen was called negative. Salk's(5) modification of the Hirst hemagglutination-inhibition test was employed in virus identification employing 4 hemagglutinating units of virus and rooster immune sera. Chicken sera were produced by injecting roosters with freshly-harvested allantoic fluid of each strain. The neutralization test consisted of equal amounts of virus containing 300 EIDs and serial dilutions of the serum. The mixture was incubated at 4°C for 4 hours and inoculated allantoically into 11-day-old embryonated eggs. The eggs were harvested after 2 days incubation at 35°C and tested with a 1% suspension of chicken red blood cells. The 50% end points were calculated by the method of Reed and Muench(6). The hemagglutination-inhibition method of Salk (5) and modified Kolmer(7) complement-fixation test were used for antibody titrations on patients' acute and convalescent sera. To determine optimal amounts of complement and virus antigen, to use in the complement-fixation test, varying quantities of each constituent were titrated together overnight in the presence of immune serum employing optimal proportions method of Friedewald(8). All complement-fixation tests were incubated overnight at 4°C before adding hemolysin and sheep erythrocytes. To remove the non-specific inhibitor before performing hemagglutination-inhibition tests, the serum was treated with receptor destroying enzyme (RDE) from *Vibrio cholerae,* employing the method of Tyrrell, *et al.*(9). Infected allantoic fluids were used as antigens in neutralization, hemagglutination-inhibition and complement-fixation tests.

Results. Clinical Observations. Thirty-two of the 37 men from whom influenza viruses, type B, were isolated had acute, febrile respiratory infections of 96 hours or less duration at admission. Four had acute respiratory infections, but temperatures less than 100°F. One man reported to infirmary for other reasons and was afebrile (98.6°F) with no respiratory complaints. Four men had throat cultures positive for hemolytic streptococci, 2 were type A19, 1 group G and 1 not typable. The total leukocyte counts ranged from 4,850 to 17,650. Oral temperature at time of nasal washing ranged from 98.6°F to 103.4°F. Twenty men were admitted to the dispensary, 10 of these were afebrile after one hospital day and all were afebrile by fifth day. Major symptoms of 36 men with respiratory complaints were malaise, cough, coryza, sore throat and headache. The signs were not distinctive. Only 8 cases were diagnosed as having influenza.

Virus isolations: Although there were scattered serological rises to influenza virus, type B, up to the middle of March, the first isolation occurred in the week Mar. 7-13. (Table

TABLE I. Virus Isolations and Serological Response.

Date	No. nasal washings	Virus isolations A	B	C	No. pairs sera	4-fold or greater antibody rises GL 1148-53A′	GL 1760-54B	GL 1167-54C
Dec.	11	0	0	2	12	0	0	1
Jan.	74	0	0	7	79	1	1	8
Feb.	226	0	0	8	213	0	14	14
Mar. 1- 6	45	0	0	1	45	0	2	2
7-13	44	0	1	1	44	0	5	4
14-20	52	0	0	0	52	0	8	4
21-27	86	0	4	2	85	0	21	8
Mar. 28-Apr. 3	86	0	8	2	86	0	22	6
Apr. 4-10	114	0	16	6	111	0	30	10
11-17	63	0	8	2	61	0	13	2

I). Isolations increased during April and in the last week of the study 8 of the 63 nasal washings yielded influenza viruses, type B. Undoubtedly, had nasal washings been collected and analyzed for viruses during the last part of April, additional strains of influenza B viruses would have been isolated. In contrast to influenza B, influenza virus type C isolations were obtained throughout the entire period of observation.

Serological observations: Only one pair of sera, obtained in January, demonstrated a significant rise to influenza A prime. Serological rises occurred to influenza B antigen in January and continued through April with highest percentage the last of March and first part of April, which coincided with the period of greatest number of virus isolations. Serological rises as well as virus isolations of influenza C occurred throughout the period of observation. The serological characteristics of the 37 strains of influenza viruses, type B, were cross-tested with rooster sera vaccinated with A (PR8), A′ (FM1), 54C (1167), and several strains of B viruses. There was no antigenic relationship to the A prime, A, or 1167-54C antisera. As seen in Table II, the 37 patients apparently had sufficient illness to provoke antibody rises as evident in homologous serum titers. These strains showed significantly less antibody rise to Lee antisera than to the 1950 and 1952 B antisera. Strain GL 1760-54B was selected as the prototype strain because it was antigenically similar to the other B viruses isolated during this period.

Table III shows the results of hemagglutination-inhibition tests employing the patients' sera against various prototype B antigens. Using the Lee B antigen, only one significant rise was obtained, whereas 29, 22, and 30 significant antibody rises were obtained respectively when 50B, 52B and 54B antigens were employed. Thus, the results using the paired sera and prototype antigens prepared from recent isolates, gave similar results to those obtained when the prototype antisera and the different isolated strains were employed as antigens (Table II). The paired sera from case No. 1756 also showed a significant hemagglutination-inhibition rise to the 54C antigen. There were no significant rises to the 53A prime virus.

With the complement-fixation test, no antibody rises were obtained with the 1940 Lee B, 50B and 52B antigens. On the other hand as seen in Table III with the 54B prototype antigen 34 of the 36 paired sera available showed significant antibody rises. The paired sera from case No. 1756 also showed a significant complement-fixation rise to prototype 54C antigen. None of the other sera showed a rise to this antigen or to the 1953 A prime antigen.

Employing sera from an individual immunized with a monovalent Lee vaccine, neutralization tests were performed with the Lee B, 52B, and 54B strains as antigens. The highest titer was obtained with the Lee antigen (5420). Correspondingly, lower titers were obtained with the 52B (512), and 54B (176).) Employing the hemagglutination-inhibition test with these antigens and corresponding sera, similar titers were obtained.

Discussion. A large proportion of the 37 cases from whom B viruses were isolated had an acute febrile respiratory illness. However,

TABLE II. Hemagglutination-Inhibition Titrations of Vaccinated Rooster Sera and Patients' Acute and Convalescent Sera for 37 Strains of Influenza Virus, Type B. Titers are expressed as reciprocal of the final dilution.

		Hemagglutination-inhibition titer of virus to:				
		Vaccinated rooster sera			Patients' homologous sera	
Nasal washings collected	GL strain No.	Lee 40B	1210 50B	704 52B	Acute	Convalescent
Mar. 12	GL 1565	32	256	256	<32	1024
22	1641	<32	256	256	<32	1024
	1645	32	128	256	<32	256
25	1671	<32	64	128	32	64
27	1701	<32	256	256	<32	1024
31	1737	<32	256	256	*	
	1739	128	512	512	<32	512
	1741	64	256	256	<32	512
	1745	64	256	256	<32	512
	1746	64	256	512	<32	1024
Apr. 1	1756	32	64	128	128	512
	1760	<32	256	256	64	256
	1771	<32	256	256	64	512
5	1803	64	512	512	128	128
	1805	64	512	512	<32	512
	1811	64	256	512	128	1024
	1814	32	256	256	32	128
	1815	32	128	256	<32	2048
	1819	32	128	256	<32	128
	1823	32	128	128	<32	1024
6	1840	64	256	512	32	512
	1845	128	512	512	128	256
8	1865	64	512	512	32	64
	1875	64	512	1024	32	64
	1876	32	128	256	32	64
	1878	32	256	256	<32	2048
9	1885	64	256	256	64	256
	1899	64	512	512	256	1024
10	1912	64	128	128	32	1024
12	1919	64	256	256	64	256
	1923	<32	128	256	64	64
	1924	64	256	256	64	512
13	1932	128	256	256	<32	256
	1938	64	256	256	64	256
	1939	32	128	256	<32	512
	1946	32	128	128	32	128
14	1954	32	128	128	32	128
Homologous serum titers:		512	1024	1024		

* No blood.

only eight were diagnosed as having clinical influenza. The problem of clinically diagnosing epidemic influenza in relation to other acute respiratory illnesses in recruits has been recently reviewed(1).

All 1954 influenza B viruses were closely related as indicated by serological procedures employed. Of special interest was the absence of complement-fixing antibodies to the 1940 Lee B, 50B and 52B strains in the patients' sera. Jensen and Francis(10) have also noted that the complement-fixation test is capable of demonstrating strain specificity. In our study the complement-fixation test could not be used to elicit the minor antigenic relationships between these 4 strains of influenza B viruses isolated in different years. However, the results of hemagglutination-inhibition tests clearly demonstrate the minor antigenic relationship between the above mentioned B viruses and therefore would appear to be a preferable technic for conducting antigenic studies.

Inhibition of agglutination with the Lee

TABLE III. Hemagglutination-Inhibition and Complement-Fixation (C.F.) Titrations of Patients' Acute and Convalescent Sera from Whom Influenza B Viruses Were Isolated.

Case No.†	Hemagglutination-inhibition titers to:* Lee 40B Acute	Conv.	1210-50B Acute	Conv.	704-52B Acute	Conv.	1760-54B Acute	Conv.	C.F. titers to: 1760-54B Acute	Conv.
1565	<32	32	32	128	32	512	<32	1024	<4	>32
1641	"	"	"	512	64	"	32	"		64
1645	64	64	"	256	32	256	<32	256		32
1671	<32	<32	"	64	"	64	"	64		16
1701	"	"	<32	512	<32	512	"	1024		32
1737	No sera available									
1739	<32	64	32	128	<32	64	32	256		8
1741	"	32	<32	512	32	512	"	1024		32
1745	32	128	32	256	<32	128	<32	256		16
1746	<32	<32	<32	"	"	512	"	512		32
1756	"	"	128	512	32	"	64	"		128
1760	"	32	32	64	"	64	32	128		64
1771	"	"	<32	1024	"	1024	"	2048		"
1803	"	<32	32	64	"	32	128	128		<4
1805	64	64	<32	128	<32	256	<32	1024		32
1811	32	32	32	256	32	512	128	"		"
1814	64	64	32	128	"	128	32	128		"
1815	<32	<32	64	256	"	512	<32	2048		128
1819	32	32	<32	128	<32	128	"	128		>32
1823	<32	"	32	256	32	512	"	1024		32
1840	32	64	"	512	"	"	"	512		8
1845	<32	<32	"	128	"	128	64	256		32
1865	"	"	"	64	"	64	32	64		16
1875	"	32	"	"	"	"	"	"		32
1876	32	"	"	"	<32	"	<32	"		"
1878	"	<32	<32	512	"	512	"	2048		16
1885	64	128	32	128	32	64	32	128		"
1899	<32	<32	<32	"	<32	"	128	1024		32
1912	"	"	"	256	"	512	32	"		>32
1919	"	"	32	128	64	128	64	512		8
1923	32	64	<32	<32	<32	<32	"	64		<4
1924	"	32	"	256	"	64	32	512		16
1932	<32	<32	32	"	32	256	<32	256		"
1938	128	128	64	"	"	"	64	"		8
1939	<32	<32	32	"	<32	"	<32	512		>32
1946	"	32	"	128	"	64	32	128		8
1954	32	"	<32	"	"	"	"	"		"
							Homologous serum titer			32

* Reciprocal of final serum dilution. † Case numbers and strain numbers are identical.

rooster immune serum to the thirty-seven 54B viruses was significantly lower than to either the 50B (VA50B), or the 52B strains. Likewise, employing acute and convalescent sera and the prototype viruses similar findings were obtained. These data show that the 1954 strains of influenza B are antigenically closer to 1950 and 1952 influenza B viruses than to Lee B strain isolated in 1940. It is also evident that employing only the Lee rooster antisera for typing, the newly isolated strains would not have been identified.[†] These observations are also borne out by the neutralization test using Lee B immune sera and these same virus antigens.

Since 1940 the influenza B viruses have been antigenically shifting away from the parent strain (Lee B), and the shift appears to be more pronounced with each epidemic, just as has been shown in the case of influenza A viruses(11-13). Because more recently isolated strains are becoming more and more unrelated immunologically to the earlier B viruses, a question has been raised by others, as well as by our findings, as to how much protection can be expected from a vaccine containing only the Lee strain of influenza B during future epidemics due to influenza B viruses(14-15). Furthermore, from our data it would appear that influenza Lee B strain is now so distantly related to recently isolated B strains that it is of little value as a diagnostic antigen. Therefore, as recommended by others, both diagnostic antigens and antisera should be prepared employing strains isolated during the most recent epidemics(16-18).

[†] Representative strains and paired sera were sent to Dr. Kieth E. Jensen, Department of Epidemiology, School of Public Health, University of Michigan, Ann Arbor, who on the basis of the hemagglutination-inhibition and absorption tests, confirmed these observations.

Summary. An epidemic of influenza B occurred in the recruit population at U.S. Naval Training Center, Great Lakes, Ill., during the Spring of 1954. Data were presented on the 37 strains isolated from individuals who were ill with an acute respiratory disease. The recently isolated influenza type B viruses are antigenically different from the 1940 Lee B. The complement-fixation test was of value only when antigens employed were prepared from the current epidemic strain of influenza B.

We wish to thank William M. Bain, John L. Langdon, Robert L. Wilkins HM3 USN, Eldon J. Kanallakan HN USN, and William M. Biringer HN USN, for their assistance. We also wish to express our appreciation to Dr. Clayton G. Loosli, and Dr. Thomas G. Ward for their helpful advice and suggestions in their roles as consultants to the Naval Medical Research Unit No. 4.

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Received February 8, 1955. P.S.E.B.M., 1955, v88.

Epidemic Influenza B and C in Navy Recruits, 1953-1954. II. Antigenic Studies on Influenza Virus, Type C.* (21610)

J. L. DeMeio, R. L. Woolridge, J. E. Whiteside, and J. R. Seal.
(Introduced by Clayton G. Loosli.)
Of the Naval Medical Research Unit No. 4, U. S. Naval Training Center, Great Lakes, Ill.

An epidemic of influenza B and C was reported in Navy recruits during the Winter of 1952 by this laboratory(1-3). During the Spring of 1954 thirty-seven influenza viruses, type B were isolated from an outbreak in the recruit population(4). Influenza virus type C was present before and during this epidemic.

This report contains studies on 31 strains of influenza virus type C isolated between the 16th of December 1953 and the 16th of April 1954 in the recruit population at this Naval Training Center.

Materials and methods. The methods for obtaining and processing nasal washings and paired sera from Navy recruits, as well as the serological methods used for virus identification, strain studies, and antibody titrations, are reported in the preceding paper(4). Influenza C antigen was prepared by inoculating 13-day-old embryonated eggs intra-amniotically and harvesting the allantoic and amniotic fluid after 4 days' incubation at 35°C. Infected eggs yielded amniotic fluid with titers of 2048 and allantoic fluid with titers of 256. The harvested fluids could not be stored at 4°C unless they were used promptly. It was found advisable to store all influenza C viruses at –70°C and thawing out only the amount of fluid necessary for immediate use. All influenza C viruses isolated in this study agglutinated chick cells but not guinea pig erythrocytes.

Results. Clinical Observations: During the period of study influenza C viruses were isolated from twenty-seven nasal washings from recruits with a variety of respiratory illnesses. Only one was considered to be characteristic of epidemic influenza. Four men, whose washings yielded influenza C viruses, had no respiratory disease symptoms. Three of these four men showed significant rises with both complement-fixation and hemagglutination-inhibition tests. The fourth man had a significant rise with the complement-fixation but none with the hemagglutination-inhibition test. Nineteen of the remaining 27 patients had significant rises with both tests, 2 had no rise with either test, 3 had no rise with the hemagglutination-inhibition test but did have a rise with the complement-fixation test. One had a rise with the hemagglutination-inhibition test, but did not have a rise with the complement-fixation test. There was no serum available from 2 patients. In none of these patients was there a correlation between the antibody level of the acute sera and the occurrence of a 4-fold antibody rise in the convalescent sera with either test.

Virus isolation and serological studies: As shown in Table I of the preceding paper, virus isolations and serological rises for influenza C were obtained throughout the entire period of study(4). There was only one antibody rise to influenza A prime while influenza B rises began in February and reached a peak in April. Virus isolations and serological rises for influenza C probably would have continued until May or later had specimens been taken.

The dates of isolation and serological characteristics of the 31 strains of influenza virus type C are summarized in Table I of this paper. There was a significant titer in each case with the 1167-54C immunized rooster serum. Strain GL 1167-54C was selected as the prototype strain when it was found to be antigenically similar to the other 30 strains

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* From Research Project, NM 005 051.06, Bureau of Medicine and Surgery, Navy Department, Washington, D.C. Views and opinions expressed herein are those of the authors and cannot be construed as reflecting the views of the Navy Department or of the naval service at large. This was also part of continuing studies on influenza made in cooperation with the Commission on Influenza, Armed Forces Epidemiological Board, and the Influenza Information Center, World Health Organization.

TABLE I. Hemagglutination-Inhibition (H.I.) Titrations of Immune Rooster Sera and Patients' Acute and Convalescent Sera for 31 Strains of Influenza C Viruses and Complement-Fixation (C.F.) Titrations to Prototype 1167-54C Strain.

		H.I. titers†			C.F. titers†	
Nasal washings collected	GL strain No.	Rooster immune sera 1167-54C	Patients' sera Acute	Conv.	GL 1167-54C Acute	Conv.
Dec, '53—16	GL 1165	2048*	128	128	4	8
17	1167	" *	64	2048	<4	64
Jan, '54— 6	1177	1024	32	128	"	8
25	1186	"	No blood			
26	1203	4096	No blood			
	1205	1024	128	512	<4	32
27	1210	512	"	1024	"	"
28	1223	2048	64	128	8	64
	1232	"	"	2048	<4	"
Feb. 2	1263	256	"	512	"	"
4	1282	512	256	256	"	<4
	1284	1024	32	128	"	16
8	1304	"	256	4096	16	256
16	1388	8192	32	128	<4	8
25	1460	"	512	2048	"	128
	1462	512	128	"	"	256
	1463	1024	"	256	"	64
Mar. 1	1484	8192	64	2048	"	16
9	1539	2048	128	256	"	32
22	1640	"	<32	"	8	64
23	1654	"	64	512	4	32
30	1734	512	"	"	<4	"
31	1743	1024	"	"	"	"
Apr. 5	1812	512	32	"	4	"
	1816	"	<32	64	32	"
6	1836	8192	64	1024	<4	"
8	1863	2048	128	256	"	16
9	1898	8192	512	4096	4	32
10	1908	2048	128	512	<4	"
12	1921	8192	512	2048	<4	16
16	1974	"	256	1024	"	"
Homologous serum titers		2048			64	

* JJ strain of influenza C used on these two tests.

† Titers are expressed as the reciprocal of the final dilution for H.I. and initial dilution for C.F. tests.

isolated. Homologous antibody titrations using the patients' virus and sera are also shown. All but six of the twenty-nine patients from whom sera were available had a 4-fold or greater homologous antibody rise by the hemagglutination-inhibition test. None of the 1954 influenza C viruses showed a significant antibody titer in the rooster immune sera to the three strains of influenza B and to the two strains of influenza A.

Also seen in Table I are the results employing complement-fixation tests on the paired sera from these twenty-nine cases using the 54-C strain as antigen. All but 3 of the patients had a significant 4-fold or greater rise to the GL 1167-54C prototype virus. These same three patients also did not show a significant antibody rise with the hemagglutination-inhibition test. Using influenza strains 52B and 53A as antigens, no complement-fixation antibody rises were observed.

Our results using GL 1167-54C and GL 1282-54C closely paralleled those obtained by Gerber, *et al.*(1) with GL 899-52C. A serological comparison of GL 1167-54C, GL 899-52C, and the 1950 JJ strain of influenza C viruses as seen in Table II show that these three C viruses, isolated in different years, are serologically the same.

Discussion: Influenza C viruses have been isolated during the seasons of 1947(6,7), 1950(5,8), 1952(1), and 1954. During the 1952 and 1954 seasons, influenza C isolations were made before, during, and after the in-

TABLE II. Comparison of 1950, 1952 and 1954 Strains of Influenza C Viruses Using the Complement-Fixation and Hemagglutination-Inhibition Test.

	Immune rabbit sera					
Virus strain	JJ C.F.*	JJ H.I.†	GL 899 C.F.*	GL 899 H.I.†	GL 1167 C.F.*	GL 1167 H.I.†
JJ—1950	128	2048	16	512	64	2048
GL 899 —1952	"	"	"	"	"	"
GL 1167 —1954	"	1024	"	"	"	"

* Expressed as reciprocal of the initial serum dilution.

† Expressed as reciprocal of the final serum dilution.

fluenza B outbreaks. While the influenza B epidemics were of 3 to 4 weeks duration, the influenza C viruses were isolated over a period of about 17 weeks.

The biological characteristics of influenza C viruses have been previously described (1,5,6). A comparison of the strains of influenza C viruses isolated in 1950(JJ), 1952 (GL 899), and 1954 (GL 1167) indicates that they have not changed antigenically insofar as can be determined by the complement-fixation and hemagglutination-inhibition tests using immune sera against the 3 strains. However, it is possible that isolates in future years will display variations just as those found among the influenza A and B viruses.

Summary. The isolation of 31 strains of influenza virus, type C, from recruits at Great Lakes Naval Training Center during the winter of 1953-54 is reported. A significant increase in hemagglutination-inhibition or complement-fixation antibody titer occurred in the blood sera of 27 of the 29 individuals from whom bleedings had been obtained. There has been no apparent change in the antigenic character of the influenza C viruses since its original isolation.

We wish to thank William M. Bain, John L. Langdon, Robert L. Wilkins HM3 USN, Eldon J. Kanallakan HN USN, and William M. Biringer HN USN for their assistance. We also wish to express our appreciation to Dr. Clayton G. Loosli and Dr. Thomas G. Ward for their helpful advice and suggestions in their roles as consultants to the Naval Medical Research Unit No. 4.

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Received February 8, 1955. P.S.E.B.M., 1955, v88.

Mechanism of Cardiac Changes Observed in Uremia.* (21611)

M. M. GERTLER, J. KREAM, AND J. HYLIN.

From Department of Cardiology, Francis Delafield Division, Columbia-Presbyterian Medical Center. (New address, Department Biochemistry, N.Y.U. Bellevue Medical Center.)

In the uremic state one frequently observes an increase in level of serum potassium and a sequential pattern of cardiac changes similar to those observed by vagal stimulation or by experimentally increasing levels of serum potassium. Since increased potassium concentration either of the heart(1,2,5) or serum (3,4) does not account for all the cardiac changes, the cholinesterase activity of hearts from uremic dogs was estimated(6-9).

Methods and materials. Studies were made on 6 normal dogs and 8 uremic dogs. Uremia was produced in 8 male dogs (16-38 kg each) by bilateral ligation of the ureters. The animals were treated symptomatically by fluid replacement and food as tolerated. Electrocardiograms were taken daily. Serum potassium was determined by flame photometry in several animals (Fig. 1). Animals were sacrificed by a cranial blow or nembutal. Rhythmic ventricular contractions were still present when hearts were removed. Blood and fat were removed from hearts prior to placing them in beakers which were immersed in an acetone-dry ice mixture to insure quick freezing. Hearts were stored in a deep freeze until used. Aliquots of ventricular muscle were cut into small portions while in a semi-frozen state and homogenized for 10 minutes with 2 volumes of ice water using a Potter-Elvehjem all glass homogenizer at 1000 R.P.M. at 2-5°C.

Enzyme activity was measured manometrically at 37° according to the method of Ammon(10). *Acetylcholine chloride* (Merck) was used as substrate in a final concentration of 0.014 M.

Results. Table I summarizes the results obtained from the assay of the cholinesterase activity of 6 heart homogenates obtained from normal animals and 8 samples obtained from animals in experimental uremia. Results are corrected for CO_2 evolved in homogenate controls and for spontaneous splitting of substrate. It is apparent that these preliminary data demonstrate a significant depression of the cholinesterase activity of homogenates derived from uremic heart samples.

Discussion. The data demonstrate a quantitative biochemical difference in cholinesterase activity as measured *in vivo* between uremic hearts and normal hearts. It must be emphasized at the onset that if depression in cholinesterase activity plays a dominant

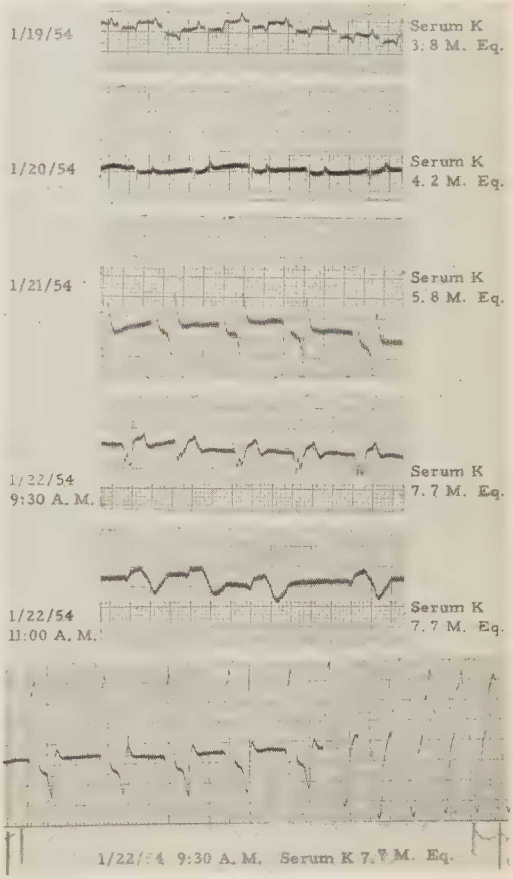

FIG. 1. Serial electrocardiogram of an uremic dog. Male. Weight 18.5 kilograms. Dates of records and levels of serum potassium are given.

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* Supported by grants from the Brook Foundation and the National Heart Institute.

TABLE I. Cholinesterase Activity of Normal and Uremic Dog Hearts.

	Q CO_2 value*	
	Normal	Uremic
	3.9	2.9
	3.5	2.1
	4.9	3.1
	4.6	2.7
	4.1	3.3
	5.6	2.6
		2.4
		3.3
Range	3.5-5.6	2.1-3.3
Avg	4.4	2.8

* μl CO_2 evolved/hr/mg dry wt of homogenate.

role in the observable cardiac changes in uremia, changes in the activity of this enzyme should be more or less exclusive and not secondary to the depression of other enzyme systems. This possibility has not been studied exhaustively.

All of the electrocardiographic abnormalities in uremia, with the exception of the T wave changes (*i.e.* incomplete heart block, complete heart block and disappearance of the "p" waves), may be explained by a depression in cholinesterase activity(7). Cardiac muscle contains both true cholinesterase and pseudo cholinesterase(12,13,14). Hence, the depression in cholinesterase activity as measured by these methods will not distinguish between the true and pseudo type of cholinesterase. However, since it has been suggested by Mommaerts(9) that the cardiac conductive elements or so-called Purkinje fibers contain mainly true or acetylcholinesterase, then it is reasonable to suggest that a depression of this esterase could result in the heart block pattern observed in uremia (11, 15,16).

Summary. 1. The cholinesterase activity of the ventricle of dogs in uremia is significantly lower than normals. 2. The observed electrocardiographic changes, characteristic of an impedance or block in action potential propagation in the heart, may therefore be specifically related to changes in the acetylcholinesterase activity of the conductive elements of this organ.

The authors wish to thank Drs. P. B. Hudson and A. L. Finkle for making the hearts of the uremic animals available for study.

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Received February 16, 1955. P.S.E.B.M., 1955, v88.

Radioiron Citrate as Tracer to Determine Disappearance Rate of Plasma Iron in Normal Subjects.* (21612)

R. Kenneth Loeffler, Donald A. Rappoport, and Vincent P. Collins.
(Introduced by R. A. Seibert.)

From Departments of Radiology and Biochemistry, Baylor University, Texas Medical Center, Houston, Texas.

Huff, Hennessy, Elmlinger and others working with Lawrence have reported studies using radioiron as a tracer to study the turnover of iron in animals, and in patients with a variety of diseases(1-5). Other groups have used this test to follow disorders of iron turnover associated with disease conditions (6-7). Generally, the tracer administered consists of globulin bound radioiron prepared from the patient's serum or using purified globulin (IV-7 fraction, Cohn)(8). The tracer is given intravenously and its rate of disappearance determined from multiple assays of plasma radioactivity. The course of iron from plasma to liver, spleen and bone marrow, can be obtained from repeated external countings over representative sites. As radioiron is incorporated in hemoglobin of newly formed red cells, it reappears in circulating blood. The percentage of tracer iron which appears in red cells is quantitatively determined at intervals of several days. The reports of Lawrence's group and others indicate the value of this test on a routine clinical basis, as an aid in diagnosis of hematopoietic disorders, as well as an index of the effect of therapy since changes in the functional status of the hematopoietic system can thus be suspected in a matter of hours, and estimated in a few days. Preparation of radioiron bound to IV-7 globulin, suitable for use in patients, has been a tedious procedure and difficult to reproduce. We have been able to prepare consistently dependable protein bound tracer only by incubating patient's plasma with radioferric chloride, buffered to pH of approximately 6 with sodium citrate, prepared just before injection(1). This requires the withdrawal of a large blood sample, centrifuging, removal of plasma, incubation with iron preparation, and reinjection, all of which must be done under sterile conditions. To obviate this time consuming procedure for each turnover, attempts were made to prepare a stock solution using either dried pooled plasma or purified IV-7 fraction obtained from Cohn's laboratory in Boston or from Cutter Laboratories in Berkeley. While several of these preparations were satisfactory, others failed to demonstrate adequate iron protein binding and gave rapid disappearance of tracer iron consistent with disappearance of injected ionized iron. In addition, there is always the problem of sterile preparation and continuous refrigeration. When stock solution is prepared from dried pooled plasma, infectious hepatitis is a serious hazard even though the likelihood is remote. Since the IV-7 plasma fraction binds the iron in undialysable form so readily *in vitro* with fresh plasma, the transfer *in vivo* may well be sufficiently rapid to permit injection of iron preparation directly to obtain a disappearance curve similar to that followed by bound material. Furthermore, since bringing ionic iron (as $FeCl_3$ in acid solution) to a pH of 6 will result in precipitation of $Fe(OH)_3$ the iron preparation must consist of iron citrate. This report gives experimental data on rate of disappearance of intravenously administered ferric citrate in patients without primary hematopoietic disorders.

Procedure. A stock solution of radioferric citrate is prepared in quantity, and transferred to sterile withdrawal bottles. The stock solution is prepared by adding $Fe^{59}Cl_3$ in HCl, with specific activity of 2000-5000 mc/g (as obtained from Oak Ridge) to 0.1% (w/v) Na citrate in isotonic saline. The solution is adjusted to a pH of approximately 6 using indicator paper, then transferred to 60

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* Supported in part by Medical Research and Development Board, Office of Surgeon General, Dept. of the Army.

ml serum bottles, and sterilized by autoclaving at 120°C for 15 minutes. The resultant solution is prepared so that initially 10 ml of solution will contain between 3 and 5 μc of Fe^{59}. Ten ml of blood are drawn into a heparinized syringe for determination of hemoglobin, Wintrobe hematocrit, red blood cell count, and a baseline determination of normally occurring radioactivity in blood, chiefly radiopotassium. Through the same needle, ferric citrate solution is administered containing approximately 4 μc of Fe^{59}, and containing from 1-5 μg iron depending on age of preparation. Five minutes after injection, another 10 ml sample of blood is withdrawn from a different vein. Two ml of this whole blood is used for determination of radioactivity, and the remainder centrifuged to obtain 2 ml of plasma for analysis of radioactivity and of iron concentration. A modification of the method of Burch *et al.*(9) is used for plasma iron concentration, normal values in this laboratory ranging from 70 to 120 μg %. At 5 minutes, and every half-hour thereafter for 4 hours, 2 ml of whole blood are withdrawn for determination of radioactivity. Radioactivity assays are determined using a well-type sodium iodide crystal with a DuMont Photomultiplier tube, operated along a plateau from 1350-1500 volts. This gives a background of approximately 250 cts/min, and a detection efficiency for Fe^{59} of about 30%. The disappearance curve is plotted on semilogarithmic paper, and plasma volume estimated from extrapolated zero-time dilution factor(1).

Experimental. Six patients with a variety of disease conditions not related directly to the hematopoietic system were chosen, on the basis of their availability for 8 hours without undergoing other procedures. Two disappearance rates were determined for each subject, first, using direct injection of ferric citrate in the morning, and second, ferric citrate incubated with plasma in the afternoon. Forty-five ml of blood were withdrawn initially for preparation of plasma, then radioferric citrate was injected along with 5 ml of Evans blue dye. Samples were taken at 5 minutes and every half hour thereafter for 4 hours. During this time an equal amount of ferric citrate

TABLE I. Disappearance Rates and Plasma Volumes Using Ferric Citrate or when Incubated with Plasma.

								Direct citrate				Incubated		
Patient	Disease	Age, yr	Ht	Wt	Hgb	Wintrobe Hct	Evans blue plasma vol	Half time	Pl vol	Isotope Hct	Iron conc., μg %	Half time	Pl vol	Iron conc., μg %
D.S.	Diabetes	18	5′ 2″	89	10.0	38	2200	37′	1740	34	32	37′	1790	40
R.M.	Carcinoma cervix	45	5′ 6″	134	10.0	38	3700	1°23′	3200	39	74	1°20′	3120	68
M.M.	Amebiasis	44	5′ 3″	116	14.1	45	2200	1°24′	2100	42	84	1°24″	2100	92
O.C.	Bartholin's cyst	28	4′11″	125	12.3	35	2200	38′	1700	32	84	38′	2060	52
D.C.	Nephrosis	28	5′ 7″	139	11.3	36	2700	42′	2300	38	60	42′	2100	36
H.S.	Stab wound	24	6′ 1″	172	13.3	39	3300	1°16′	3100	35	56	1° 7′	2960	60

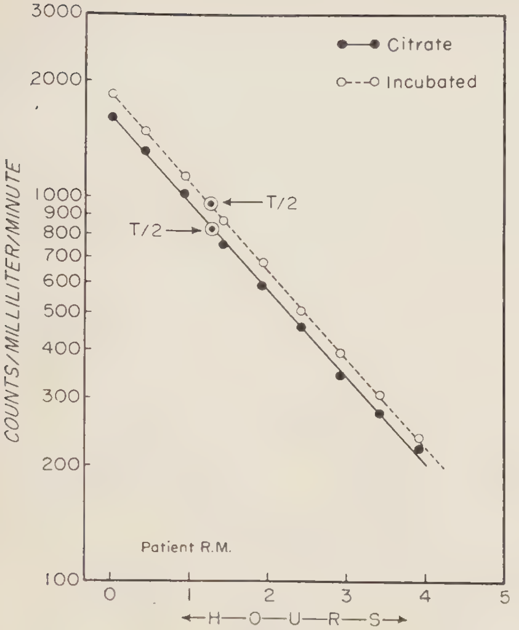

FIG. 1.

was added to the separated plasma, and incubated at room temperature. Four hours after injection of ferric citrate, the incubated plasma was injected, and again blood samples taken at 5 minutes and at half hourly intervals for 4 hours. The results, demonstrating similarity of behavior of the two forms of iron, are presented in Table I. Urine samples were collected on the first three subjects, and contained less than 0.5% of injected radioiron administered as citrate, with no excretion of radioiron following injection of incubated plasma. Urine of fourth subject was collected at one minute intervals through ureteral catheters during the first hour of ferric citrate injection. All radioisotope excretion occurred during the first 2 minutes. These studies are being continued on patients having a wide variety of disease conditions, and utilizing other iron preparations. It may be questioned whether the iron citrate technic would be valid for use with patients having a very high serum iron as in hemochromatosis or hemosiderosis. Assuming that there is an absolute amount of iron binding protein available, a serum iron level of 300 μg % may approach saturation. Then the tracer dose of iron citrate could not be incorporated with protein bound iron. Data of Gitlow(10,11) indicate that saturation in excess of 95% will rarely be encountered. Five % unsaturation of a binding capacity of 300 μg % would represent an ability of protein to chelate an additional 150 μg/l of plasma. The tracer dose of 1-5 μg should not be excessive even in these clinical conditions, though we have not yet had a patient of this category for study. To date, the citrate disappearances have been identical to those of the globulin-bound iron. These studies will be submitted as a separate report when the general applicability has been better established.

Conclusions. Radioferric citrate, administered intravenously to patients without primary disorder of hematopoiesis, disappears from the plasma at a rate similar to that of the disappearance of globulin-bound radioiron. It is probable that the iron is rapidly incorporated into the iron binding globulin of the plasma (IV-7 fraction, Cohn), and the disappearance rate obtained following the injection of ferric citrate is actually that of the disappearance of protein bound iron. The use of ferric citrate as a tracer for iron turnover studies is a simplification which permits

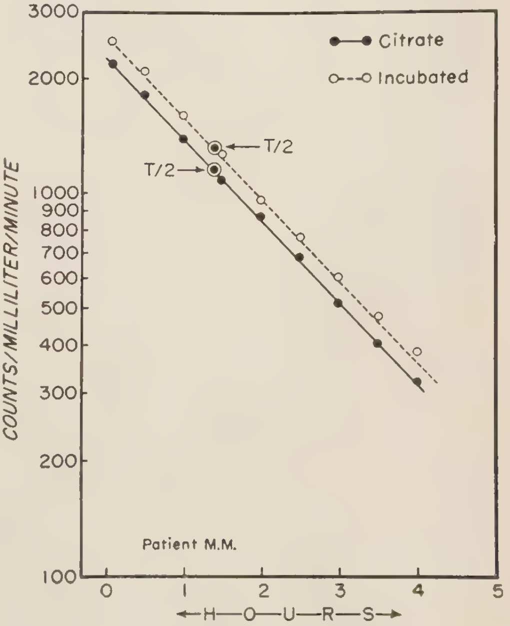

FIG. 2.

the more routine availability of these studies in a clinical practice.

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Received February 17, 1955. P.S.E.B.M., 1955, v88.

Maintenance of Pregnancy in Absence of Dietary Protein with Progesterone.* (21613)

Marjorie M. Nelson and Herbert M. Evans.

From the Institute of Experimental Biology, University of California, Berkeley.

Nelson and Evans(1) have recently shown that pregnancy can be maintained in 100% of the animals on a protein-free diet by daily injections of a combination of estrone (0.5-1.0 μg) and progesterone (4 mg), whereas a daily dosage of 4 mg of progesterone alone maintained pregnancy in 70% of the animals. To determine whether this progesterone dosage was optimal for the experimental conditions, levels of this hormone varying from one to 8 mg have been tested for maintenance of pregnancy in the absence of dietary protein.

Methods. Stock female rats of the Long-Evans strain, 80-85 days of age and averaging 190-195 g in body weight, were bred with normal males and placed on the protein-free diet the first day of gestation. The protein-free diet, the same as that used in previous studies (1,2), consisted of 88% sucrose, 8% hydrogenated vegetable oil (Crisco or Primex), and 4% salts. Crystalline vitamins per kilogram of diet were: 50 μg vit. B_{12}, 600 μg d-biotin, 10 mg 2-methyl-1, 4-naphthoquinone, 10 mg thiamine HCl, 10 mg pyridoxine HCl, 11 mg pteroylglutamic acid, 20 mg riboflavin, 20 mg p-amino-benzoic acid, 50 mg niacin, 100 mg d-calcium pantothenate, 800 mg inositol, and 1.0 g choline chloride. All rats received weekly a fat-soluble vitamin mixture containing 800 U.S.P. units vit. A, 115 chick units vit. D, 6 mg dl-alpha-tocopherol, and 650 mg corn oil. (Mazola). *Progesterone injections* were started 3 days after breeding and continued until the day before autopsy. Synthetic progesterone was dissolved in sesame oil and 0.1-0.5 cc injected subcutaneously once per day. Ten to 12 animals were used for each of the following progesterone levels, namely 1, 2, 3, 4, 6 and 8 mg. Four groups, 2 receiving 0.3 cc sesame oil daily and 2 without any injections, were kept as controls. Vaginal smears were examined daily during gestation for the presence of erythrocytes, the

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* Aided by grants from College of Agriculture of University of California, Roche Anniversary Foundation, and U. S. Public Health Service. We are indebted to Dr. Daniel A. McGinty of Parke, Davis and Co., Detroit, Mich., and to Chemical Specialties, Inc., New York City, for generous supplies of progesterone; to Dr. T. H. Jukes, Lederle Laboratories, Pearl River, N. Y., for pteroylglutamic acid and vit. B_{12}; to Dr. Elmer L. Sevringhaus of Hoffmann La Roche, Inc., Nutley, N. J., for d-biotin, d-calcium pantothenate and dl-alpha-tocopherol; and to Dr. Randolph Major, Merck and Co., Inc., Rahway, N. J., for crystalline B vitamins and 2-methyl-1,4-naphthoquinone.

TABLE I. Effect of Varying Levels of Progesterone on Reproduction of Rats Maintained on Protein-Free Diets.

Progesterone dosage (mg daily)	Rats bred (No.)	Wt change during gestation (g)	Onset vaginal RBC[*] (day)	Resorptions (%)	Litters[†] (%)	Implantation sites/rat (No.)	Living fetuses/litter[‡] (No.)
Series 1—Animals autopsied day 13 of gestation							
0	20	–30	10.0	89	11	9.4	
1	12	–38	10.7	83	17	8.3	
2	12	–36	11.6	42	58	9.2	3.7
3	10	–31	12.4	40	60	11.3	10.5
4	10	–30	13.0	20	80	8.7	5.9
6	11	–29	12.5	0	100	10.6	8.9
8	12	–25	11.5	8	92	10.5	7.0
Series 2—Animals autopsied day 21 of gestation							
0	20	–41	10.2	95	5	5.7	
1	12	–40	11.4	83	17	8.8	
2	12	–39	11.3	75	25	10.1	6.7
3	12	–36	12.7	67	33	10.1	6.5
4	20	–31	12.1	30	70	8.9	6.2
6	10	–29	13.2	40	60	9.8	6.3
8	10	–24	12.0	30	70	10.0	5.7

[*] Day when vaginal smear first contained erythrocytes (RBC).

[†] This column gives the % of rats with living fetuses at autopsy; this does not imply that the rats littered, inasmuch as the animals were autopsied before parturition in both series of experiments.

[‡] Values are given only for groups in which at least 3 rats had living embryos or fetuses at autopsy.

sign that implantation has occurred. Two series of experiments were carried out. In the first series, the animals were autopsied on day 13 of gestation, and in the second series on day 21, one day before the expected parturition.

Results. Table I summarizes the data on reproductive performance for the 2 series of experiments. In the *first series,* in which all rats were autopsied on the 13th day of pregnancy, the maintenance of pregnancy increased as the progesterone level was raised to the 6 mg daily dosage. This progesterone level resulted in living fetuses in 100% of the animals at autopsy. Regardless of the dosage, there was no significant difference in the number of implantation sites per rat. The early appearance of vaginal erythrocytes on the 10th to 11th days, indicating fetal death and beginning resorption of one or more implantation sites(3), occurred in some groups.

In the *second series,* in which all animals were autopsied on the 21st day of pregnancy, maintenance of pregnancy improved as the progesterone level was increased to the 4 mg daily dosage. This hormone level resulted in living fetuses in 70% of the animals at autopsy. Higher levels, 6 or 8 mg daily, did not prove to be more beneficial.[†] The maintenance of pregnancy resulting in all groups in this series was less than that obtained for corresponding groups of the first series autopsied earlier in gestation. When pregnancy was maintained with progesterone the number of living young per litter averaged 6.2 in contrast to the normal average of 9.1(1). At higher levels of progesterone the rats tended to lose less weight during the gestation period. This may have been due to water retention, as such animals exhibited marked edema of the abdominal organs, especially in the region of the pancreas. A similar edema has been noted previously in protein-depleted rats in which pregnancy was maintained by the combination of estrone and progesterone(1).

Discussion. Lyons(4) has shown that the combination of estrone and progesterone maintained pregnancy in the absence of the pituitary and the ovaries, whereas either progesterone or estrone given separately had no effect. The maintenance of pregnancy by injection of progesterone alone in the protein-depleted rat would, therefore, appear to indicate the presence of some circulating estrin in these rats. The difference in reproductive performance in groups receiving the same progesterone level but autopsied on the 21st day instead of the 13th day of pregnancy may have been due to a progressive decrease in circulating estrin as protein depletion continued. An alternative explanation would be an increase in the estrin requirement for the latter part of pregnancy. Apparently protein depletion during pregnancy affected estrin production only gradually but had an immediate effect on progestin production, so that progestin was the principal hormonal deficiency under these experimental conditions.

[†] Lack of deleterious effects on reproduction shown by higher levels (6-8 mg) of progesterone in both series of experiments should be noted. Levels higher than 8 mg daily were not tested because of possible anesthetic effects. In contrast, high levels of estrone (4-6 μg) exhibited markedly injurious effects on reproduction(1).

The level of progesterone required to maintain pregnancy in a majority of the protein-depleted rats appeared to be 4 mg daily. For the first 13 days of pregnancy, the highest percentage of litters resulted from the use of the 6 mg daily dosage. No data are available in regard to the progestin requirement of the hypophysectomized - oophorectomized rat for the first 13 days of pregnancy in comparison with that for the latter part of pregnancy. It is possible that placental production of luteotrophin may change the requirement during the latter part of the gestation period. The slightly better reproductive performance during the first 13 days obtained with the 6 mg progesterone level in comparison with the 4 mg level in these protein-deficient rats is similar to that observed with the same hormone dosages in thiamine-deficient rats for the same period(5).

In hypophysectomized - oophorectomized rats the requirement for progesterone must be determined in combination with estrone, inasmuch as progesterone alone is insufficient for maintenance of pregnancy in these animals. Lyons(4) reported that pregnancy was maintained to a greater extent in the doubly-operated animals receiving 4 mg progesterone daily than when the 3 mg daily dosage was used together with estrone. The protein-deficient rats showed little difference in reproductive performance for the 3 and 4 mg progesterone levels during the first 13 days of pregnancy. By the 21st day, however, maintenance of pregnancy was significantly better in the group receiving the 4 mg daily dosage. The remarkable similarity in the level of progesterone required by both protein-deficient and hypophysectomized-oophorectomized rats suggests the absence of circulating progestin in animals deprived of dietary protein during pregnancy.

Summary. Levels of progesterone, varying from one to 8 mg daily, were given to rats of the Long-Evans strain receiving a protein-free diet throughout the gestation period. Under the experimental conditions the level of progesterone required to maintain pregnancy in a majority of the animals closely approximated 4 mg daily.

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Received February 18, 1955. P.S.E.B.M., 1955, v88.

Serum Cholesterol, Phospholipid and Lipoprotein Levels in Elderly Male Subjects. (21614)

P. G. Ackermann, H. J. Buehler, G. Toro, and W. B. Kountz.

From St. Louis Chronic Hospital, Division of Gerontology, Washington University School of Medicine, and the Central Research Laboratory, Anheuser-Busch, Inc., St. Louis.

In connection with studies on serum lipoproteins in elderly individuals(1,2) the serum cholesterol, phospholipid and S_f 12-20 levels were determined in elderly male subjects. All tests were not run on all subjects, but the total numbers of satisfactory subjects used for tests were sufficiently large (60 to 80) to warrant reporting the results.

Methods. Subjects were inmates of the St. Louis Chronic Hospital, aged 53 to 94 years. Subjects with acute or infectious diseases, nephrosis, diabetes, extreme hypertension or recent (within one year) myocardial infarction or cerebrovascular accident were excluded from the tests, as were subjects suffering from definite malnutrition. S_f values were determined by the method described by De Lalla and Gofman(3). Cholesterol and lipid phosphorus were determined by methods given earlier(2) and lipid phosphorus converted to phospholipid by the empirical factor of 25. All determinations were made on serum from blood samples drawn in the morning before breakfast.

Results. Table I gives a comparison of the S_f 12-20 levels with age of subjects. In this only average value was used for each subject. Results are not included for any subject with a history of recent medication with thyroid or sex hormones. Subjects were divided into age groups and the average S_f values computed for each group. There is a statistically significant difference in averages only between oldest and other groups. For all subjects, however, the correlation coefficient between age and S_f 12-20 level is $r = -0.348 \pm 0.129$ (significant at the 1% level). This negative coefficient is in agreement with the work cited by Keys(4) in which values for 61-70 age group averages lower than the 51-60 age group. We determined S_f levels in a few young individuals and obtained results in the range of 15 to 20 mg %. These are in accord with values of Milch(5) who found S_f 12-20 levels of 20 mg % in a number of males in the 20-25 age group which increased to approximately 40 mg % in the 40-45 age group. Recently Glazier and associates(6) have published data on the S_f values in a large number of subjects. In young male subjects their average level of S_f 12-20 lipoproteins was approximately 40 mg % with average levels up to 60 to 70 mg % in older subjects. Our values as tabulated are somewhat lower. These authors did find that the levels for male subjects in the 60 to 70 age group were on the average slightly lower than those for the 50 to 60 age group.

TABLE I. Correlation of S_f 12-20 Levels with Age.

No. of subjects	Avg age	Avg S_f 12-20 level (mg %)
10	54.6 (53-58)	45.3 ± 3.4*(22-63)
13	64.6 (60-68)	41.8 ± 4.7 (21-87)
23	74.9 (70-79)	41.7 ± 3.7 (26-64)
14	86.5 (80-94)	31.7 ± 4.7 (12-76)

* Stand. dev. of the mean.

Figures in parentheses show range of values found.

TABLE II. Correlation of Serum Cholesterol and S_f 12-20 Levels.

No. of subjects	Avg cholesterol level in mg %	Avg S_f 12-20 level in mg %	Avg age
25	199.2 (160-228)	28.5 ± 1.4*(16-50)	72.4
27	242.8 (230-258)	41.6 ± 2.8 (22-60)	74.3
28	285.4 (260-327)	43.0 ± 2.4 (13-63)	73.1

* Stand. dev. of the mean.

Figures in parentheses show range of values found.

Table II shows results of comparison of the S_f 12-20 levels and cholesterol levels. In the comparison of cholesterol levels with S_f or phospholipid levels, some results are included from patients with previous medication when their serum lipids appeared to be stable at the determined levels. The data were divided into 3 nearly equal groups on the basis of cholesterol levels as indicated in the Table and the average S_f levels computed for each group.

The last column of the Table gives average age of each group and demonstrates that there is no age difference between groups. There is an increase in average S_f value with the cholesterol level and a statistically significant difference between the first 2 groups. For all subjects the correlation coefficient between cholesterol and S_f levels is $r = +0.564 \pm 0.077$ (significant at the 0.1% level). Though the correlation is good and in agreement with the work of Gertler and Oppenheimer(7) who found a coefficient of 0.56, there is a considerable spread of S_f values for each group.

A third correlation was made between cholesterol and phospholipid values in serum. Peters(8) found a linear relationship between serum cholesterol and phospholipid, which on converting his lipid phosphorus values to phospholipid by the empirical factor of 25, is equivalent to the equation: $P = 0.735\,C + 90$ where P is the phospholipid level and C the cholesterol level both expressed in mg %. In the analysis of 104 pairs of cholesterol-phospholipid values by the method of least squares we found the equation: $P = 0.783\,C + 73$. The difference between constants of the 2 equations is not significant. They are valid only over the range from 150 to 450 mg %, the 2 equations give calculated phospholipid values differing by only a few mg % for the same cholesterol value. At 200 mg % cholesterol these equations lead to cholesterol:phospholipid ratios of 0.846 and 0.870 respectively and at 300 mg % cholesterol the ratios are 0.970 and 0.975. These equations which are derived from experimental data indicate that the average cholesterol:phospholipid ratio varies with the cholesterol concentration. It has been suggested(9) that this ratio may be an indicator of deranged lipoprotein equilibrium. Since this ratio varies with the cholesterol concentration, either the latter should be as good an indicator or it would be more logical to compare the measured cholesterol ratio not with a ratio derived from a cholesterol level of say 180 mg % but with that obtained from the actual cholesterol level using one of the above equations.

Summary. The cholesterol, phospholipid and S_f 12-20 levels in the blood serum were determined in a number of elderly subjects all over the age of 52. In these age groups there was a slight decrease in S_f level with age and a good correlation between the S_f level and the cholesterol level. The phospholipid level was found to vary linearly with the cholesterol level.

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Received February 21, 1955. P.S.E.B.M., 1955, v88.

Glycogen in Skin of the Mouse During Cycles of Hair Growth. (21615)

MARGARET SHIPMAN,* HERMAN B. CHASE,† AND WILLIAM MONTAGNA.‡
(Introduced by Paul F. Fenton.)
From Arnold Biological Laboratory, Brown University, Providence, R. I.

There is a general misconception that the amount of glycogen in skin of mammals remains constant. Those who have studied it histochemically, however, are aware of the close relation which exists between amount of glycogen in skin and the growth cycle of hair follicles. For this reason, a study of glycogen in skin in relation to controlled phases of hair growth cycle is of interest.

The epidermis of newborn mammals is rich in glycogen but the epidermis of the adult contains none(3,14,18). In adult skin, glycogen can be demonstrated in the epidermis only during wound repair and in pathological conditions of the skin(1,5,7). Glycogen normally occurs in growing hair follicles(4,10,11,16,17), and its presence or absence there is correlated with the hair growth cycle. This paper traces histochemically and biochemically the rise and fall of glycogen in the hair follicle during the hair growth cycle.

Materials and methods. This study was carried out on a large series of C57 Black (C57BL/6Ch) mice. Growth of hair was initiated by plucking the club hairs(6) and pieces of skin were removed at 0, 1, 2, 3, 4, 5, 6, 9, 16, 18, 19, 20, 21, and 22 days after plucking. The specimens were taken at 11 A. M., fixed in Zenker-formol and embedded in paraffin. Five micron sections were stained with the periodic-acid Schiff technic of McManus(15). Alternate sections, incubated in buffered diastase(12) for one hour at 37° or for 10 minutes in saliva served as controls. For determination of *glycogen,* three pieces of skin were removed from each animal at 3, 6, 9, 16, and 22 days after plucking. Each sample of skin was weighed and hydrolyzed in potassium hydroxide. Glycogen was precipitated in 95% ethyl alcohol. After redissolving in water, glycogen was determined by the anthrone method(19).

Observations. During *anagen I* (1-2 days after plucking) cells of the quiescent follicle go into mitotic activity and proliferate downward into the dermis. During *anagen II* (2-3 days after plucking) a cord of cells extends downward and encloses the ball of papillary cells. The inner root sheath begins to be formed as a thin keratinized dome just above the cone of epidermal cells around the papilla. During this time, a few granules of glycogen appear in cells of capsule beneath the resting club of the previous hair generation. There is no other visible glycogen in the hair follicle at this stage. *Anagen III* (3-4 days after plucking) is the period during which the whole follicle grows downward and establishes its characteristic size and shape. In the middle segment of the outer sheath, the periphery of cells is outlined by granules of glycogen. The inner sheath begins to plow its way upward and at the end of this period it extends to the level of the sebaceous gland. The first pigmentary dendritic cells appear in the bulb.

Anagen IV (about 6 days after plucking) marks the beginning of actual hair production. The base of the follicle has reached a depth of about 500 μ below skin surface. A hair, with cortex and medulla is formed. Glycogen is present in cells of middle segment of outer sheath from above the bulb to just below the sebaceous duct. Glycogen is also present in the nonkeratinized cells of the medulla and cuticle of the cortex. At the end of anagen IV the tip of the hair is at the level of sebaceous gland. *Anagen V* (8 days after plucking) is that stage when tip of hair reaches the skin surface. Cells of the middle portion of external sheath are laden with glycogen.

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* Submitted in partial fulfillment of requirements for the M. S. degree at Brown University.

† Work supported by a grant-in-aid from the U. S. Public Health Service and by a grant-in-aid from the American Cancer Society upon recommendation of the Committee on Growth of the National Research Council.

‡ Work supported by a grant-in-aid from the U. S. Public Health Service.

By *Anagen VI,* critical formative stages have occurred and hair continues to be formed for a period of 8-9 days. During this period the outer sheath remains rich in glycogen and small amounts may be found in the unkeratinized cells of the medulla and cuticle of the cortex. In the medulla of the hair, glycogen is particularly conspicuous in the lower middle portion of the follicle.

Catagen (about 17 days after plucking) is the transitory period which marks cessation of hair growth. A brush-like club and a surrounding capsule are formed. The lower one-half to two-thirds of the follicle, including the bulb degenerates, the dermal papilla is released and forms a ball of cells at the base of the abortive, quiescent follicle. At the end of catagen, the base of the follicle is about 250 μ below the skin surface. Visible glycogen disappears suddenly from the entire follicle.

Telogen is the resting stage of a follicle and it is reached approximately 19 days after plucking. Resting follicles contain no visible glycogen.

In the one-day-old mouse, development of the hair follicles is dischronous; whereas some follicles may be no more advanced than epidermal buds, others have progressed to anagen II, III, or IV. Each individual follicle follows a pattern of growth identical to that of the adult mouse(6); the distribution of glycogen in the follicle is also similar to that described above for adult mouse.

Unlike that of older animals. the surface epidermis contains abundant glycogen. One day after birth, all layers of the epidermis, except the stratum corneum are rich in glycogen. On the second day, the epidermis has less glycogen and basal cells are free of it. The amount of glycogen in epidermis diminishes gradually until 6 days after birth, at which time most follicles are in anagen VI. Nine days after birth the epidermis no longer shows demonstrable glycogen.

From the biochemical analysis, it is clear that there is some variability in amount of glycogen from the three samples taken at the same time from the same animal, but overall results show a remarkably consistent trend. The average gammas of glycogen per mg of wet skin at the indicated days after plucking are as follows: 3 days, 1.071; 6 days, 1.498; 9 days, 2.002; 16 days, 2.312; 22 days, 0.712. The skin during periods of hair production contains 2 to 3 times as much glycogen as the skin during telogen or early anagen. It is to be noted also that at catagen glycogen values drop suddenly. Although a certain proportion of the glycogen in each of these values must represent glycogen in the muscle of the panniculus carnosus, there is a close parallelism between the biochemical findings and the visual observations.

Discussion. During the resting period and early periods of mitotic activity, there is little or no glycogen in the hair follicle. Glycogen is gradually accumulated in the follicle as the structure of the active hair follicle is reestablished. During the period of hair production, glycogen is present in large amounts in the external sheath of the middle segment of the follicle, and in the unkeratinized cuticle of the hair shaft and medulla. This increase in amount of skin glycogen can be detected biochemically as well as histochemically.

The distribution of glycogen suggests that there is an inverse relationship between the storage of glycogen and cell proliferation and differentiation(16). When cells are actively dividing or when they are becoming rapidly keratinized, glycogen may be used up rather than stored; in cells where keratinization is slow and less complete, such as in the cuticle of the cortex and the medulla, glycogen can be demonstrated. This point is also illustrated by injured epidermal cells which neither divide nor undergo complete keratinization. At this time they are also full of glycogen(2, 5,7,13). The distribution of glycogen in the skin of the newborn mouse also illustrates these points. It is well known that fetal epidermis, which shows sluggish and incomplete keratinization abounds in glycogen(5,9,18).

Glycogen in hair follicles may represent a nutritive source for growth and regeneration. Perhaps, the growing hair follicles store large amounts of glycogen so that keratinization, which takes place some distance from the dermal blood supply, may have a store of glycogen for anaerobic glycolysis(5).

In the early stages of anagen the increasing amounts of glucose available may be used for energy by the cells in mitosis. During anagen I and II, there is no glycogen present. During anagen III, mitotic activity wanes in the external sheath but it increases in the bulb and glycogen begins to be stored in the middle portion of the sheath. Growth of the hair shaft begins in anagen IV and mitotic activity becomes confined mainly to the bulb. It is possible also that the cells of the outer sheath, which are practically at a standstill mitotically, no longer need glycogen for energy; and since the blood supply is still high, glycogen is stored in them. This supply of glycogen could be used during active hair formation. In catagen, glycogen disappears quickly as the vascularization is decreased (8).

Summary. During telogen, anagen I, and anagen II no glycogen is present in the hair follicle except for occasional granules in the capsule. Glycogen begins to appear in the lower external sheath in anagen III and persists in large quantities in this layer during the period of hair production. In addition, glycogen is found in the unkeratinized cuticle of the hair shaft and medulla in anagen IV through VI. Glycogen disappears abruptly from all these structures during catagen. Skin has 2 to 3 times as much glycogen during periods of hair growth than it has during telogen or at three days after plucking (anagen stages preceding hair growth). Cytochemical data indicate that this increase is due largely to the increased amount of glycogen found in the external sheath during periods of active hair production.

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Received February 21, 1955. P.S.E.B.M., 1955, v88.

Inhibition of Renal Tubular Responsiveness to Antidiuretic Hormone By Pyrogens.* (21616)

J. L. Brandt, H. D. Ruskin, B. Zumoff, L. Castleman, and S. Zuckerman.
From Department of Medicine, State University of New York, College of Medicine, Brooklyn, New York City.

In the course of studies(1) of the diuresis associated with the administration of large intravenous doses of bacterial pyrogens, data were obtained which suggested that this material specifically interfered with the renal tubular response to antidiuretic hormone. The following experiments were devised to test this postulate.

Methods. Under light pentobarbital anesthesia, each of 10 normally hydrated, previously untreated, fasting female mongrel dogs were given an intravenous infusion of 5% glucose in water at a constant rate of 5 cc to

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* This work is being supported by a grant from the American Heart Assn. and the Orange County Heart Assn.

10 cc per minute. Ten to 20 minute collections of urine from the bladder were made during the development of water diuresis and throughout each experiment, using an indwelling catheter. Following the establishment of the water diuresis at a constant level for 3 or 4 periods, antidiuretic hormone (ADH, vasopressin, Pitressin®) was added to the infusion. The rate of ADH infusion was also constant throughout each study, and ranged between 25 and 100 milliunits per hour in different experiments. Following the establishment of a constant antidiuresis for 3 or 4 periods, 50 micrograms of bacterial pyrogen (Piromen®-5 cc)[†] were injected into the infusion tubing, and urine collections were continued for 2 to 3 hours. In several animals, single intravenous doses of 5 units of Pitressin were given at the height of a pyrogen diuresis induced by the injection of 50 μg of bacterial pyrogen. Heparinized blood samples were drawn at the beginning and end of each experiment involving a water diuresis, *e.g.* in the 10 animals cited above. Urinary and plasma solute concentrations (osmolarity) were measured by freezing point depression, and total solute excretion per minute was calculated from the product of urine flow and urine solute concentration. A number of the animals were restudied about a week later to evaluate renal concentrating and diluting function by means of water deprivation and forced water diuresis respectively.

Results. It soon became apparent that for dogs in which a water diuresis was established and then inhibited by ADH, followed by a large dose of pyrogen, a reproducible set of responses could be anticipated. A typical response for one dog is tabulated in Table I and demonstrated in Fig. 1. The response in all animals was a uniform rise in urine flow to a maximum level and a corresponding fall in urinary solute concentration as water diuresis became established. Urine osmolarity was high before the water diuresis and fell approximately 90% with the increase in urine flow during water diuresis, so that total solute excretion remained fairly constant. With the administration of ADH there was a fall in urine flow to 0.5 cc/min. or less and a corresponding increase in urinary solute concentration, total solute excretion remaining unchanged. (There was a transitory fall in solute excretion for one collection period after the start of Pitressin antidiuresis, see Fig. 1). Following a delay of approximately 40 minutes after the injection of pyrogen there was an abrupt rise in urine flow with a corresponding fall in osmolarity again maintaining solute excretion per minute near initial levels, except for a slight transient increase in solute excretion at the onset of the pyrogen diuresis. The increasing urine flow reached a peak about 30 minutes after the onset of the pyrogen diuresis and remained at that level for one or two collection periods after which it fell slightly but continued high during the next two hours. At all times, with peak diuresis, urine osmolarity was well below that of plasma. The latter tended to fall slightly between the begining and end of the study. The animals, in this group, which were restudied were found to have normal concentrating and diluting function.

TABLE I. Water Diuresis-Pitressin Antidiuresis-Pyrogen Diuresis in the Dog.

Time (min.)	Urine flow (cc/min.)	Urine osmolarity (mOsm./l)	Total solute excretion (μOsm./min.)
0	5% glucose/water infusion-rate—6 cc/min.		
110-120	3.20	105	336
120-130	3.55	100	355
130-140	3.55	106	376
140	Pitressin added to infusion—100 mU/hr		
140-150	3.63	110	399
150-160	1.00	211	211
160-180	.72	512	370
180-190	.45	892	402
190	Piromen—50 γ I.V.		
190-210	.34	1104	375
210-230	.40	1008	406
230-240	.85	702	597
240-250	2.00	252	504
250-260	3.65	146	533
260-270	3.95	104	411
270-280	3.15	106	334
280-300	2.25	131	295
300-330	2.67	112	299

In the animals in which ADH was given at the height of the pyrogen diuresis (see meth-

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[†] Piromen was supplied by Travenol Laboratories, Morton Grove, Ill. This material is a nonprotein complex polysaccharide derived from pseudomonas. The material used contained 10 γ/cc.

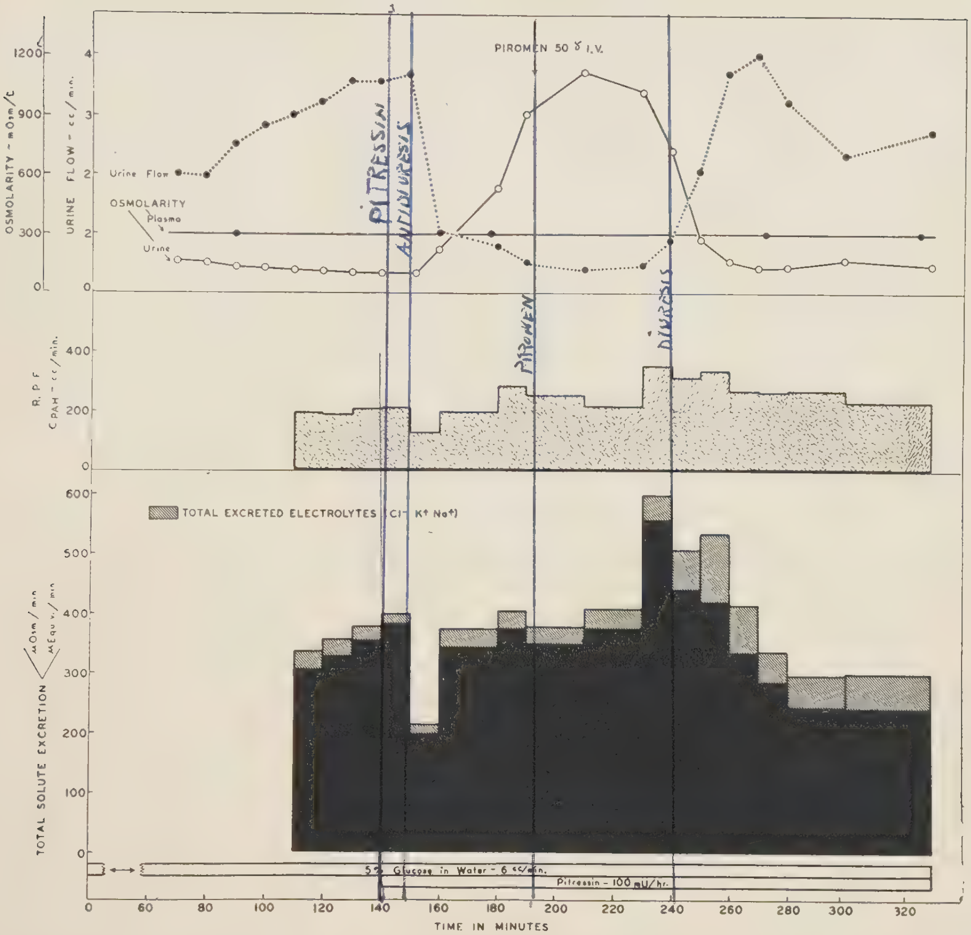

FIG. 1. Relationship of urine flow, renal plasma flow, urine and plasma osmolarity, and urine solute excretion during sequence of water diuresis, Pitressin antidiuresis, and pyrogen diuresis. Note presence of continuous Pitressin infusion during pyrogen diuresis.

ods) it was noted that no antidiuretic effect developed in spite of the presence of pressor and other systemic effects.

Discussion. The present experiments have clearly demonstrated that a large dose of bacterial pyrogen abolishes the responsiveness of the renal tubule to antidiuretic hormone in the dog. The doses of pyrogen used do not permanently damage tubular functions of concentration or dilution. The uniform association of an increased renal plasma flow with the diuresis evoked by the pyrogenic reaction (1,2) is probably not related to the diuresis. No consistent relationship exists between plasma flow and urine flow(3) under normal circumstances. The relative constancy of total solute excretion during the development of pyrogen diuresis and the presence of marked hyposmolarity of the urine at peak flow eliminate an increase in glomerular filtration rate as the important factor accounting for increased urine flow associated with pyrogens. Tubular responsiveness to ADH is altered by pyrogen whether this is given before or after the pituitary hormone. Apparently pyrogens inhibit ADH and alter tubular handling of water by a specific effect within the kidney for systemic effects of pitressin are evident when given in large doses. Histological evidence of cloudy swelling confined to the distal tubule has been reported in guinea pigs after large doses of pyrogen(4). *

Whether these histological changes can be correlated with biochemical changes and the alterations of tubular function induced by pyrogens remains to be seen. Pyrogen may prove to be a useful tool in elucidating some of the problems presented by diabetes insipidus, and the normal ability to elaborate dilute or concentrated urine.

Summary and conclusions. 1. In the dog a single large intravenous dose of pyrogen completely abolished the renal response to antidiuretic hormone whether it was given before or after the pituitary hormone. 2. No permanent alterations of concentrating or diluting function of the kidney could be demonstrated for the doses of pyrogen used.

The authors wish to express their thanks to Audrey Jones and Mary Bianchi for technical assistance.

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Received February 28, 1955. P.S.E.B.M., 1955, v88.

Fate of Intravenously Administered Glucosamine in the Rat. (21617)

NORMAN F. BOAS* AND JOSEPH B. FOLEY.

From the National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Public Health Service, U. S. Department of Health, Education and Welfare, Bethesda, Md.

Hexosamines† are widely distributed throughout all tissues of the body(1), and exist almost entirely as components of mucoproteins, nucleic acids and mucopolysaccharides. To study the manner in which the body utilizes glucosamine, the free compound was administered intravenously to rats and its rate of disappearance from blood and distribution in body tissues studied.

Methods. Male Sprague Dawley rats were individually caged, fed a stock diet(2) prior to the experiments, and permitted to drink water *ad libitum*. All rats were fasted 24 hours before the start of the experiments. Using ether anesthesia, animals were "hepatectomized" by ligating the hepatic artery and portal vein at the hilus of the liver and "nephrectomized" by ligating the renal vessels close to the hili of the kidneys. Unneutralized glucosamine-HCl (4% or 20% solution) was injected intravenously in the external jugular vein in a volume of 0.25 ml/100 g body wt at the completion of each operation, or when no operation was performed, at the start of an experiment. Tail blood samples were collected and mixed in spot plates with dry oxalate crystals prior to the injection of glucosamine and at various time intervals following the injection. In the tissue study, the rats were killed by exsanguination under ether anesthesia, after obtaining a sample of tail blood. The tissue samples and whole blood samples from all experiments were hydrolyzed in 2N hydrochloric acid for 15 hours at 100°C. Total body hexosamine was determined by hydrolyzing a whole rat in 6N hydrochloric acid for 5 hours at 100°C. Hexosamine was isolated from the diluted and filtered hydrolyzates using columns of Dowex-50, and determined colorimetrically by a modification of the method of Elson and Morgan(1).

Results. Blood Hexosamine. In the first set of experiments glucosamine was injected intravenously and the rate of its disappearance from the blood was measured. Intact rats weighing 90-130 g were used as well as those in which the liver or kidneys had been excluded from the circulation by ligating their

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* Present address: Department of Laboratories, Norwalk Hospital, Norwalk, Conn.

† Natural occurring hexosamines are glucosamine and galactosamine. The method used here does not distinguish between these two amino sugars.

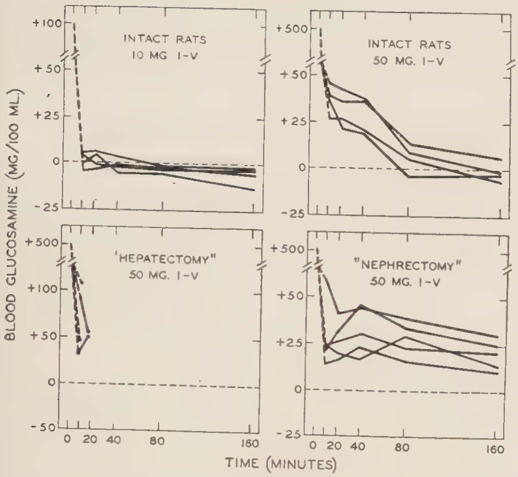

FIG. 1. Rate of disappearance of intravenously administered glucosamine from blood of intact rats and rats with ligated hepatic or renal blood vessels. Dosage shown is in mg/100 g body wt. Curves are based on changes in total blood hexosamine from observed pre-injection control values. Each curve represents a serial study on an individual rat.

major vessels. In the first experiment intact rats were injected with 10 mg of glucosamine-HCl/100 g body wt. Assuming a normal blood volume of approximately 10% of body weight(2), this should have been sufficient to increase the blood hexosamine level 100 mg % above the normal blood level. All the data are presented relative to an initial control blood level (varied from 54-81 mg %) prior to the time of injection (Fig. 1). As can be seen, 95% or more of the injected hexosamine disappeared from the blood within the first 10 minutes after injection. In the second experiment rats were injected with 50 mg of glucosamine-HCl/100 g body weight. This was sufficient to theoretically increase the blood levels 500 mg %. Again, over 90% of the injected hexosamine disappeared from the blood within the first 10 minutes and was entirely gone at the end of 160 minutes. In order to evaluate the possible role of the liver in removing hexosamine from the circulation, a group of "hepatectomized" rats were injected with 50 mg of glucosamine-HCl/100 g body wt. Most of these animals went into shock and died after 10-20 minutes, however, blood levels obtained as early as 10 minutes after the injection demonstrated that, again, 80-90% of the hexosamine had disappeared from the blood. In considering renal excretion as a factor, a group of "nephrectomized" rats were injected with 50 mg of glucosamine-HCl/100 g body wt. The values at 10-40 minutes were in the same range as those observed in the similarly treated intact rats. The values did not return to normal at 80 and 160 minutes as in the intact rats, but remained at approximately 25 mg %. The intact kidneys, therefore, were able to excrete or destroy about 5% $\left(\frac{25}{500}\text{ x }100\right)$ of this administered dose of hexosamine.

Blood incubation. In view of the very rapid disappearance of hexosamine from the blood, incubation studies were carried out to see if blood itself was destroying the hexosamine. Recovery experiments were run in which glucosamine-HCl had been added to fresh defibrinated whole blood. The mixtures were incubated at 37°C for 10 minutes, and hexosamine determinations made. All of the added hexosamine was recovered, indicating no evident destruction in the short period of time that it takes for the body to remove hexosamine from the blood.

Distribution in tissues. In order to study the fate of intravenously injected glucosamine in tissues, two 120 g rats were used, a control rat and one which was injected with 45 mg of glucosamine-HCl. A tail blood sample was obtained 11 minutes after the injection. Both rats were sacrificed by exsanguination, the injected rat, 18 minutes after the injection. Most of the major tissues and organs were sampled and their hexosamine concentrations determined. The concentration of hexosamine in a given tissue is ordinarily very constant from rat to rat.‡ As can be seen in Table I, the concentration increased in every tissue examined. Donaldson's data(3) on body composition of the rat were used to estimate the distribution of the administered dose of hexosamine. All of the tissues studied represented approximately 75% of the total body

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‡ This has been true in all tissues which have been studied in groups of animals. *E.g.* whole blood 68 ± 4 mg/100 ml; liver 64 ± 5 mg/100 g; total carcass (98 g rats) 79 ± 5 mg/100 g.

TABLE I. Distribution of Hexosamine in Tissues after Intravenous Injection in Rat.

		Hexosamine, mg/100 g			Administered glucosamine	
Tissue	% of body wt*	Control	Injected	Change	Total mg‡	% distribution
Skeletal muscle	41.0	23	35	+12	5.9	12.8
Blood	7.0	73	103	+36	3.0	6.5
Loose connective tissue	15.0†	88	122	+34	6.1	13.2
Liver	6.0	71	121	+50	3.6	7.8
Brain	1.7	79	88	+ 9	.2	.4
Testes	1.3	45	50	+ 5	.1	.2
Stomach and intestines	1.2	127	179	+52	.6	1.3
Kidneys	1.0	122	194	+72	.7	1.6
Lung	.6	93	111	+18	.1	.2
Spleen	.3	67	75	+ 8	.03	.1
Total	75.1				20.33	44.1

* Based on data of Donaldson(3).

† Estimated.

‡ Total mg = (Change, mg/100 g) × (body wt) × (% of body wt).

weight. The integument and bones, which were not examined, constituted the remaining 25%. The kidneys, intestines and liver demonstrated the greatest increase in concentration. In terms of total distribution, however, the largest amount of the administered dose went to loose connective tissue (13.2%), muscle (12.8%), liver (7.8%), and blood (6.5%). Only 44% of the administered dose could be accounted for by the tissues examined.

Recovery of hexosamine from carcass. To determine whether or not the body destroyed free hexosamine, total carcass analyses of hexosamine were carried out on groups of 65-90 g intact rats which had received intravenous injections of 50 mg of glucosamine-HCl/100 g body wt. There were 6 rats in each group. The rats which received glucosamine were killed with ether at 20, 80 and 320 minutes following the injection. The excreta were collected and added to the rat prior to hydrolysis of the whole animal. The total body hexosamine values of the injected rats were compared with those of an uninjected group. The recovery of hexosamine from the injected group was compared with the recovery of glucosamine which had been run through the whole procedure separately. The mean recovery values for hexosamine from the injected rats after 20, 80, and 320 minutes were 77 ± 11%, 39 ± 11%, and 29 ± 8%, respectively. It was thus evident that after one hour, over 50% of an injected dose of hexosamine could be destroyed in the body. This could in part be explained by the instability of glucosamine under neutral pH conditions (Table II). Three 20% solutions of glucosamine-HCl were permitted to stand for varying lengths of time at room temperature at different pH conditions. The glucosamine was shown to be stable at acid pH but was slowly destroyed under neutral or alkaline pH conditions. On the basis of this data, one would not expect a 10 minute incubation of glucosamine with blood to effect any detectable destruction.

Discussion. When free glucosamine is administered to the rat intravenously it disappears very rapidly from the blood. It has been demonstrated that glucosamine given either intravenously or subcutaneously to rabbits will appear in the urine(4,6). It would appear, however, that this represents only a small fraction of the total administered dose, since this rapid disappearance also occurs in rats with ligated renal vessels. Intravenously administered glucosamine appears to distribute itself in large part in those tissues which are rich in extra-cellular fluid (muscle, and

TABLE II. Effect of pH on Stability of Glucosamine-HCl.

	Destruction (%)		
pH	2 hr	3 hr	24 hr
2.9	0	0	0
7.4	11	13	40
8.6	11	—	45

connective tissue), but with the greatest concentration increases appearing in liver, kidney and in the intestines, a finding in agreement with the observations of Kawabe(6). Many of these earlier studies on the fate of parenterally administered glucosamine, however, are difficult to interpret because of the non-specificity of the hexosamine methods used(4,6). The liver did not seem to play a significant role in explaining the rapid disappearance of hexosamine from the blood, in the work reported here, since the blood levels were essentially the same in intact animals, as in those in which the liver was excluded from the circulation.

Analyses of total carcasses following the injection of glucosamine clearly demonstrated that as much as 50% of the administered dose may be destroyed within 1 hour of injection. This may be partly explained on the basis of instability of glucosamine at neutral body pH.

Summary. 1. Following the intravenous administration of glucosamine-HCl to the rat, 80-90% disappears from the blood within the first 10 minutes, in intact animals, as well as those in which the liver or kidneys have been excluded from the general circulation. 2. No destruction is evident when glucosamine is incubated with whole blood for 10 minutes. Glucosamine is destroyed, however, under neutral or alkaline pH conditions after a more prolonged period of time. 3. Intravenously administered glucosamine-HCl becomes rapidly distributed in all tissues of the body, with the greatest concentration increases appearing in the kidneys, liver, and intestines. In total amount, however, most of the administered dose is found in muscle, connective tissue, blood and liver. 4. Approximately 50% of intravenously administered glucosamine-HCl is destroyed in the body in a period of less than 1 hour.

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Received November 22, 1954. P.S.E.B.M., 1955, v88.

Toxicity of Histamine and Antagonism between Histamine and Antihistamines in Various Strains of Mice. (21618)

J. L. AMBRUS, P. S. GUTH, S. GOLDSTEIN, M. E. GOLDBERG, AND J. W. E. HARRISSON.
(Introduced by J. M. Coon.)

From the LaWall Memorial Laboratory of Pharmacology and Biochemistry, Philadelphia College of Pharmacy and Science.

Several authors have reported that antihistamines do not protect mice against the toxic effects of histamine and may even show synergistic action(1-3). In a previous study (4) it has been shown that regarding the toxic and body temperature decreasing effects of histamine in mice, antihistamines may be synergists, antagonists or may have no effect, depending on doses and ways of administration. In the latter study it was reported that 5 mg/kg NN-Dimethyl-N′ (P-methoxy-benzyl)-N′-(2 pyridyl) ethylenediamine maleate (pyrilamine maleate, Neoantergan) protected mice against subcutaneous toxic doses of histamine. Deutsch and Loew(5) repeated the latter experiment using a similar schedule and found no protection.

It appeared to be of interest to investigate the reasons for the discrepancy of the above results. Since different mouse strains and different weight ranges were used in the two studies, the toxicity of histamine and antagonism between histamine and antihistamines were investigated in various inbred and not inbred strains of mice.

Materials and methods. Females of the

TABLE I. LD_{50} of Subcutaneously Administered Histamine in Various Strains of Mice without and with Neoantergan Pretreatment.

Strain	Sex	Wt range	No. animals	LD_{50} histamine (base), mg/kg	LD_{50} histamine (base), mg/kg after 5 mg/kg Neoantergan	% protection
Swiss (ICR)	♀	25 ± 3	220	230	1560	+578
" Webster (Tac.)	♀	22 ± 4	370	785	1328	+ 69
Idem	♀	30 ± 5	110	899	1155	+ 28
DBA/2	♀	20 ± 5	44	1209	1634	+ 35
C_3H/Jax.	♀	23 ± 5	48	1523	1358	— 11

following strains of mice were used: DBA/2, C_3H/Jax, Swiss Webster (origin: Taconic farms). In addition, results obtained with Swiss (ICR) mice in the previous study(4) are included. Breeders of the latter strain were kindly supplied by Dr. T. Hauschka from his colony and were maintained in this laboratory. Histamine diphosphate U.S.P. was injected subcutaneously; doses reported are calculated to the base. Neoantergan* was injected subcutaneously 20 minutes preceding histamine. Animals were observed for 24 hours following injection. Results reported are the composite of several experiments performed on different days. LD_{50} was calculated using the method of Behrens *et al.*(6). This method was selected since the number of animals in the various groups as well as the spacing of doses was not equal. This was due to the dependence on breeding schedules and the limited number of animals of the desired strain, sex, and weight range available on any one day.

Results. Results are summarized in Table I. It appears from these data that the higher the natural resistance of a mouse strain to histamine, the less protection is afforded by pretreatment with Neoantergan. In Swiss Webster mice, resistance increases with increasing age and weight. Accordingly, protection by Neoantergan decreases. This is not due to higher Neoantergan requirement for antagonizing higher lethal doses of histamine, since higher doses of Neoantergan actually showed synergism with histamine(4). Similarly, some synergism was observed between histamine and antihistamines in the most histamine resistant mice (C_3H). The same relationship is evident if species of various sensitivity to histamine are compared. The highly histamine sensitive guinea pigs can be protected to the greatest extent, while in the histamine resistant mice and rats less protection is evidenced. When histamine sensitivity of mice is increased by adrenalectomy(7) or pertussis vaccinations(8-11), protection again occurs with antihistamines.

Discussion. All these findings suggest that the toxicity of histamine in mice may be manifested through two different mechanisms. The action on one is antagonized by antihistamines. This is the mechanism which is less operative in naturally more histamine resistant strains. The effect on the other mechanism is not affected by antihistamines. Loew (12) suggested previously that the synergism between the intravenous toxicity of histamine and antihistamines in mice may be due to the fact that acidity is an important factor in view of the high doses used. Deutsch and Loew(5) found no synergism when neutralized histamine diphosphate was employed. A double mechanism is also suggested by the data of Munoz, Schuschardt and Verwey(13-14) who found that in mice sensitized to histamine by adrenalectomy or pertussis vaccination, a greater percentage of mice survived higher doses of histamine than those receiving lower doses. The same authors noticed marked differences between various strains of mice in the extent of sensitization to histamine after adrenalectomy. Fink and Rothlauf(15) compared the sensitivity of various strains of mice to a standard dose of histamine and to anaphylaxis.

Summary. 1. Sensitivity to histamine of four strains of mice has been compared. 2. Within one strain older and heavier mice appeared to be more resistant than younger and lighter ones. 3. The more histamine sensitive a strain or weight-range of mice proved to be, the more protection was observed by pretreatment with an antihistamine. 4. It is suggested that the toxicity of histamine in mice may be manifested through two different mechanisms.

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* Kindly supplied by Dr. R. Paul of Rhone Poulenc Co.

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Received January 18, 1955. P.S.E.B.M., 1955, v88.

Lipides of Chick Heart Fibroblasts. Effect of Vitamin B_{12} and Folic Acid.* (21619)

BENJAMIN W. GRUNBAUM, FREDERICK L. SCHAFFER,† AND PAUL L. KIRK.
(Introduced by F. W. Allen.)

From Department of Biochemistry, University of California, Berkeley.

Tissues cultured *in vitro* deteriorate in the presence of old medium, or after prolonged existence without being subcultured. One of the first symptoms of this deterioration is the formation of fat globules in the cytoplasm. In the phenomena associated with deterioration with age, a quantitative study of fat metabolism of tissues showing this effect seemed desirable. The authors have previously(1) described a method for fractionation of 0.2 to 1 mg amounts of Earle's strain L cells. In the work presented here, that method was used for the study of the effect of vit. B_{12} and folic acid upon the lipides of chick heart tissue cultured *in vitro*.

Methods and procedure. Methods and procedure detailed previously(1) for fractionation of tissue and subsequent quantitative analysis were followed. The *technic of culturing* chick embryo heart was similar to that described by Signorotti *et al.*(2). All the media mentioned are described elsewhere(3) in detail. Hearts of 11½-day-old chick embryos were cut into 16 approximately equal pieces which were implanted into 4 roller tubes, 4 fragments to the tube. The fragments were embedded in 0.2 ml of 20% chicken plasma and appropriate medium added. Incubation was carried out in a rotor in the conventional manner. *Separation of Outgrowth from Explant.* Because of the difficulty in obtaining a "pure" outgrowth, the whole culture(4), including explants and clot are often used for quantitative chemical analysis of tissue culture. A substantial uncertainty is thus admittedly introduced. A

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* This investigation supported by grants from the National Cancer Institute of the National Institute of Health, U. S. Public Health Service, and the University of California Committee on Research.

† Present address: Virus Laboratory, University of California, Berkeley.

TABLE I. Effect of Vit. B_{12} and Folic Acid on Total Nitrogen and Relationship of Total Fatty Acids to Lipide Phosphorus.

Medium*	Exp. 1 μMFA/μMP†	γN	Exp. 2 μMFA/μMP†	γN	Exp. 3 μMFA/μMP†	γN
Standard	1.95	35	2.09	58	2.18	14
Standard + folic acid	1.95	41	1.84	57	1.80	58
Standard + Vit. B_{12}	1.80	45	1.81	87	1.93	62
Standard + folic acid & Vit. B_{12}	1.80	46	1.86	89	1.85	69

* A standard medium consisting of 20% embryo extract (1:1 dilution embryo juice and Tyrode's solution), 40% horse serum, and 40% Tyrode's solution, gave dense growth characterized by spindle shaped and clear cells.

† μM fatty acids/μM phosphorus.

procedure for separation of outgrowth from tissue implanted, was developed as detailed below. The liquid medium was removed and clot washed with 0.9% saline and drained. With a simple hoe-shaped tool made of 2 mm stainless steel wire, the explant was excised and removed. The remaining tissue and clot were drawn together and separated from the glass. A few milliliters of saline were added, shaken lightly and the entire contents transferred to a 15 ml centrifuge tube. Pooled outgrowths, usually from 6 cultures were centrifuged, the saline decanted and 0.025% trypsin in Tyrode's solution added. Trypsin treatment was added to the procedure to dissolve the clot and other sources of extraneous nitrogen such as dead cells and cellular debris. Trypsin treatment was carried out with stirring by a magnetic stirrer for 2 hours at 37°C at pH 7.8-8.0. The remaining pellet of outgrowth fibroblast cells was washed twice with saline and prepared for analysis as previously described for strain L cells(1).

Results. Preliminary experiments indicated that vit. B_{12} and folic acid exerted a definite effect on growth of cultures. Accordingly, stock solutions of these materials were made in Tyrode's solution and added to cultures at a final level of 0.4 μg/1 ml of medium. Three sets of similar experiments were made, the results of which are shown in Tables I and II, along with comparative values for chick heart tissue at the time of excision, with and without trypsin treatment, and for 6-day-old explants.

Each value in Table I represents total nitrogen of 24 individual pieces of tissue, obtained by pooling tissue outgrowths in 6 roller tubes, to minimize errors in cutting and variations in amounts of outgrowth. Radial outgrowth with standard medium in Exp. 2 was the same as that of vitamin treated tissues, but was markedly less dense, as reflected by low nitrogen value. In all cases, cultures in standard medium served as controls with which the treated tissues were compared. Although considerable variability among experiments was noted, within each experiment higher nitrogen values were observed in those cultures treated with both vitamins. This is taken as indication of growth stimulation by vitamins. In Table I, the relation of fatty acid to phosphorus is presented independently of nitrogen. Rather constant values were observed for vitamin treated tissues, with consistently higher values for controls.

In Table II are shown comparative values for lipide content of cultured chick embryo heart tissue and a comparison with values obtained from freshly excised untreated heart

TABLE II. Comparative Mean Values for Lipide Content of Chick Embryo Heart Tissue.

Type of tissue	Fatty acid, μM/mg N	Lipide P, μM/mg N	M fatty acid / μM P
F*	4.17	2.00	2.07
F treated with folic acid	3.01	1.62	1.86
F treated with vit. B_{12}	3.11	1.68	1.83
Idem & folic acid	2.49	1.32	1.84
Freshly cut chick embryo heart 11.5 days old	2.55	1.36	1.84
Idem, trypsin treated	2.64	1.45	1.80

* F = Fibroblasts.

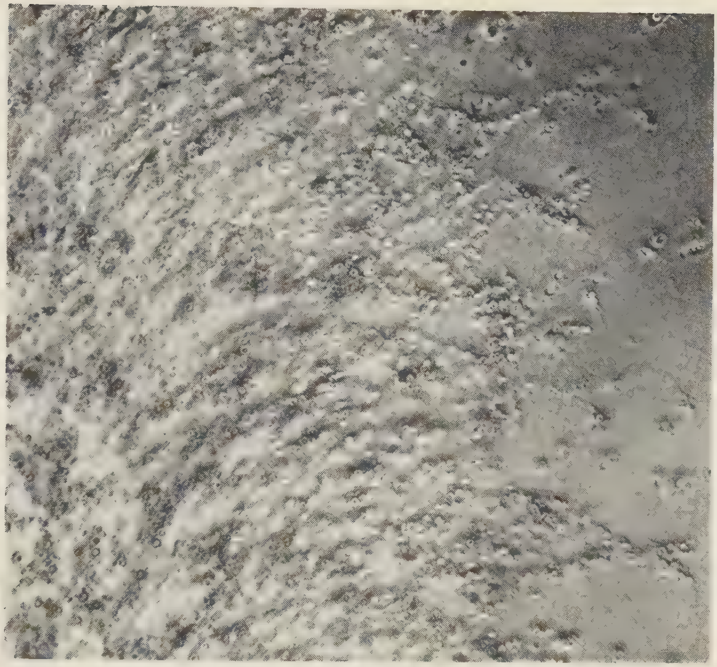

FIG. 1. Eight-day-old fibroblasts from chick embryo heart grown on standard medium. Phase microscope photograph (× 180).

tissue, and that treated with trypsin. Addition of vit. B_{12} and folic acid resulted in considerable reduction in both fatty acids and phospholipide phosphorus relative to nitrogen content. The similarity of amount of lipides in tissues grown in media containing the two vitamins to that in original tissue suggests a role of the vitamins in maintaining relative lipide content of original embryonic tissue. The fact that control and treated tissues were both healthy in appearance and showed cell division may be related to the approximate constancy of ratio of fatty acids to phospholipide phosphorus, shown in Table I. It is not clear whether the slightly higher values for tissue grown in standard medium are to be considered as abnormal, but addition of an older horse serum to the medium or excision after more than 6 days led to still higher ratio of about 2.8. In comparison with the ratio with vitamin treated tissues, a value of 2.8 appears to be definitely abnormal.

Effect of Vit. B_{12} and Folic Acid on Aging Tissue. Fig. 1 shows an 8-day-old control culture which has deteriorated with rounding of the cells and extensive fat accumulation. In Fig. 2 is shown a culture of identical age and culture conditions except that vit. B_{12} and folic acid were added to the medium. During the first 3 days, growth of the control (Fig. 1) appeared to be more rapid than that of treated tissue (Fig. 2). On the fourth day no difference between the cultures could be observed, after which treated tissue continued to grow normally and the control developed degenerated condition associated with old cultures. The culture shown in Fig. 2 survived for several additional days after it was photographed.

Fat accumulation has long been associated with degenerative changes that occur in old cultures. Results presented here indicate that a definite relation exists between fat accumulation and degeneration and death of the cells, because when accumulation is prevented by vitamins, the other aspects of degeneration also do not occur.

Discussion. The apparent effect of vit. B_{12} and folic acid was to increase the amount of metabolizing tissue by a stimulation of protein synthesis accompanied by a decreased relative lipide content.

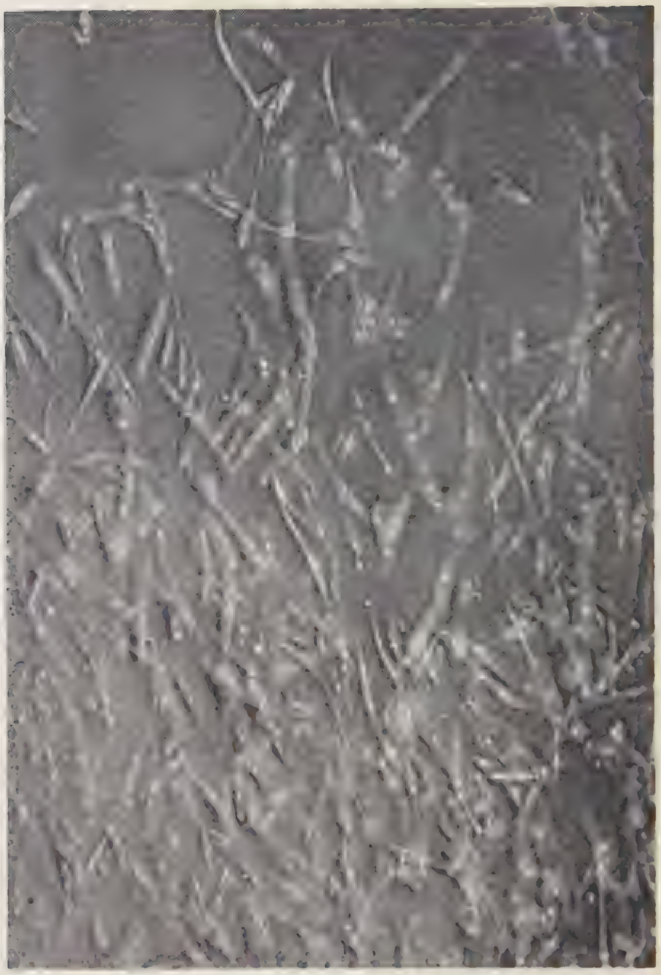

FIG. 2. Eight-day-old fibroblasts from chick embryo heart grown on standard medium plus vitamin B_{12} and folic acid. Phase microscope photograph (× 180).

The ratio of fatty acid to phospholipide phosphorus in growing tissue, which remained constant at about 2:1, seems to indicate that lipides were almost entirely phospholipides. Maintenance of this ratio coincided with the period of normal, rapid growth and mitosis of chick heart fibroblasts. The ratio changed rapidly soon after maximum area was attained by the culture. When embryonic chick heart tissue was grown for one or more days longer than those reported above, the ratio of fatty acid to phospholipide phosphorus increased, indicating an increase in neutral fat. Efforts to study the chemical changes in the cells of a degenerating culture were not wholly successful because no adequate evaluation could be made of the amount of metabolizing tissue. Trypsin treatment followed by centrifuging led to formation of a layer composed largely of neutral fat on top, and a layer of tissue cells on the bottom of the solution. The bottom layer showed analyses similar to explant tissue but probably did not include rapidly degenerating cells, which may not have been trypsin resistant. Further efforts to study this question were currently discontinued.

The experiment illustrated in Fig. 1 and 2 shows that addition of vit. B_{12} and folic acid prevented deterioration of cultures for the time interval studied. It is postulated that they delayed aging phenomenon by stimulating protein synthesis and preventing deficiencies in mechanisms involved in fat metabolism.

Definite interpretation of results of studies on metabolism of tissue is almost impossible at present because of the complex materials employed such as plasma, serum, and embryo extract. In contrast, advances in the study of metabolism by means of bacteria have been made more readily because cultures were pure and synthetic media were used. The work presented in this study is an approach to studies which may lead to a better understanding of aging phenomena of tissue *in vitro* and *in vivo*.

Summary. 1. A study of amount and nature of lipides that accumulate in "old" cultures of chick heart fibroblasts was made, along with the effect of vit. B_{12} and folic acid on the accumulation. 2. Vit. B_{12} and folic acid in the medium prevented accumulation of fat and apparently stimulated growth of the culture. Normal controls, freshly excised tissue, and vitamin treated cultures were essentially free of neutral fat as indicated by constancy of ratio of fatty acids to phospholipide phosphorus. In degenerating cultures this ratio increased rapidly in the absence of added vitamins.

The authors wish to acknowledge the excellent technical assistance of Mrs. Elsa Zitcer, tissue culturist, and Mrs. Shirley Moss, analyst.

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Received January 18, 1955. P.S.E.B.M., 1955, v88.

Maternal Vitamin B_{12} Deficiency and Nucleic Acid Content of Tissues from Infant Rats.*† (21620)

JOSEPH H. BRUEMMER, BOYD L. O'DELL, AND ALBERT G. HOGAN.
From Department of Agricultural Chemistry, University of Missouri, Columbia.

Hydrocephalus has been observed in infant rats as a result of a maternal deficiency of folic acid(1,2) and of vit. B_{12}(3). Histological examination(4) has revealed that the hydrocephalus was associated with an abnormal cerebral aqueduct which in some cases was occluded at birth and in all cases was abnormal in size and shape. In a series of embryonic brains from vit. B_{12} deficient dams, the group of columnar ependymal cells normally found in the roof of the cerebral aqueduct and third ventricle was completely or partially missing(5). Thus, it is apparent that a maternal deficiency of vit. B_{12} interferes with the development of the fetal brain, and this may be related to nucleic acid metabolism since the nucleic acid composition of tissue has been shown to be affected by a deficiency of vit. B_{12}. The pentose nucleic acid content of the livers of weanling and of adult rats is decreased as shown by chemical (6,7) and cytochemical(8-10) methods.

A study was made of the nucleic acid composition of tissues from newborn rats produced by deficient dams. The composition of brain tissue received major attention, but for comparative purposes liver tissue was also analyzed. The brain tissue from the vit. B_{12} deficient offspring contained more nuclei as well as more deoxypentose nucleic acid (DNA) per gram than tissue from the controls so that the amount of DNA per nucleus was unchanged. The pentose nucleic acid (PNA) per cell was less in brain tissue from deficient animals, but the difference in liver tissue was not significant.

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* Published with approval of Director of Missouri Agric. Exp. Station, Journal Series No. 1468. This investigation supported in part by a grant from the National Institutes of Health, U. S. Public Health Service.

† This is a portion of thesis to be submitted to Graduate School by J. H. Bruemmer in partial fulfillment of the requirements for the Ph.D. degree.

Methods. Female rats were depleted of vit. B_{12} by maintaining them from weaning to maturity on a vit. B_{12} deficient ration(11), of the following percentage composition: soybean oil meal 70, cerelose 22, lard 4, and salts mixture(1) 4. The following vitamins[‡] were added per kilo of ration: thiamine HCl 16 mg, riboflavin 16 mg, calcium pantothenate 40 mg, pyridoxine HCl 16 mg, choline chloride 1 g, biotin 0.2 mg, folic acid 5 mg, alpha-tocopherol 30 mg, 2-methyl-1, 4-naphthoquinone 10 mg, vit. A 20,000 I.U. and vit. D 2,850 I.U. The offspring of dams fed the basal ration supplemented with 30 μg of vit. B_{12} per kilo were used as controls. The experimental animals for nucleic acid analysis were selected from litters which had at least one case of hydrocephalus in the same litter or in a previous litter. After decapitation, the brain or liver was quickly excised, chilled on cracked ice, blotted free of excess moisture, and weighed. A 5% homogenate of the tissue was prepared using cold 0.1 M citric acid and a chilled glass homogenizer(12). The number of nuclei in the homogenate was determined by use of a leucocyte diluting pipette and a hemocytometer. The diluting fluid was 0.1% toluidine blue in 3% acetic acid. The proteins were precipitated from the cold homogenate by the addition of cold trichloroacetic acid to give a final concentration of 8%, and the nucleic acids were extracted by the method of Schneider (13). DNA was determined by the diphenylamine method of Seibert(14), and PNA by the method of Albaum and Umbreit(15). Commercial DNA[§] ($N = 1.62\%$; $P = 9.5\%$; $E^{1\%}_{1\,\mathrm{cm}} = 226$) and commercial PNA[||] ($E^{1\%}_{1\,\mathrm{cm}} = 232$; ribose $= 19.6\%$ using a ribose standard (15)) were used as standards. Blood urea nitrogen was determined by the method of Barker(16) and glucose by the method of Nelson(17). Liver glycogen was isolated by the procedure of Good *et al.*(18), and determined as glucose by the method of Nelson (17).

Results. The effectiveness of the depletion achieved in the experimental animals used in this study is attested by the occurrence of hydrocephalus in a high percentage of the litters and by other gross symptoms. Usually the offspring failed to survive more than 2 or 3 days, and before death exhibited the gross symptoms of dyspnea, cyanosis, weakness and emaciation. Schultze(19) described similar symptoms associated with acute uremia in newborn rats from vit. B_{12} deficient dams. Although the newborn animals observed in this study had nursed, considerably less milk was present in their stomachs than was normally found in the control animals.

Besides the gross symptoms, determinations of blood urea, blood glucose and liver glycogen were also used as criteria that a vit. B_{12} deficiency existed in the newborn rats produced under the experimental conditions. The results of these determinations are given in Table I. The blood urea nitrogen of vit. B_{12}-deficient animals 36-72 hours of age was almost 3 times the average value obtained on controls of the same age. There was not a significant difference in the blood glucose concentration between the deficient and control groups, but the average liver glycogen of the vit. B_{12} deficient animals was about one-fifth that of the controls.

The effect of a vit. B_{12} deficiency in the maternal diet on the nucleic acids of the brain and liver is shown in Table II. The average

TABLE I. Blood Urea Nitrogen, Blood Glucose and Liver Glycogen of Newborn Rats.[*]

Supplement	Blood urea nitrogen (mg/100 ml)	Blood glucose (mg/100 ml)	Liver glycogen (%)
0	92.0 ± 4.9[†] (26)[‡]	102 ± 17 (6)	.39 ± .12 (6)
Vit. B_{12}	36.3 ± 3.3 (16)	88 ± 4 (8)	2.02 ± .24 (6)

[*] Urea nitrogen was determined on animals 36-72 hr of age; glucose and glycogen on animals 24 hr of age.

[†] Stand. error of mean.

[‡] No. of animals analyzed shown in parentheses.

[‡] The folic acid supplied through the courtesy of Dr. T. H. Jukes, Lederle Laboratories, Pearl River, N. Y., and the other crystalline vitamins through the courtesy of Dr. H. H. Draper, Merck and Co., Rahway, N. J.

[§] Nutritional Biochemicals Corp., Cleveland, O.

[||] Pfanstiehl Chemical Co., Waukegan, Ill.

TABLE II. Effect of a Maternal Deficiency of Vitamin B_{12} on the Nucleic Acid Content of Brains and Livers from Newborn Rats.*

			DNA		PNA	
Supplement	Tissue wt (mg)	Nuclei/g ($\times 10^6$)	Per g (mg)	Per nucleus (mg $\times 10^{-9}$)	Per g (mg)	Per nucleus (mg $\times 10^{-9}$)
			Brain			
0	167 (23)†	530 ± 12‡	4.12 ± .11	7.8 ± .2	2.97 ± .11	5.6 ± .2
Vit. B_{12}	203 (20)	468 ± 6	3.56 ± .06	7.7 ± .1	3.28 ± .07	7.0 ± .2
		$P<.01$§	$P<.01$	$P>.05$	$P<.05$	$P<.01$
			Liver			
0	210 (15)	671 ± 22	5.27 ± .19	7.9 ± .2	9.79 ± .23	14.7 ± .5
Vit. B_{12}	255 (14)	637 ± 21	4.89 ± .22	7.7 ± .2	10.28 ± .35	16.3 ± .5
		$P>.05$	$P>.05$	$P>.05$	$P>.05$	$P<.05$

* Avg age of newborn animals for brain analyses was 20 hr and for liver analyses, 5 hr.

† No. of animals averaged in parentheses.

‡ Stand. error of mean.

§ P values indicate significance of the difference between groups with and without vit. B_{12} as determined by the "t" test of Fisher.

age of the animals used for the brain analyses was 20 hours and for the liver analyses, 5 hours. Some animals in the deficient group were hydrocephalic, but they did not differ significantly from their litter mates and were included in the average. A vit. B_{12} deficiency increased the amount of DNA per gram of brain from 3.56 to 4.12 mg per gram. The number of nuclei per gram was also increased by the vit. B_{12} deficiency, and as a consequence, the average amount of DNA per nucleus was the same for deficient and control groups. The amount of PNA per gram of brain and per nucleus was decreased by the vit. B_{12} deficiency. The difference between the amounts of PNA per gram of brain was not highly significant, but the amount per cell was 20% less in the deficient animals ($P<0.01$). There was no difference in the content of moisture and nitrogen in the brain tissue from the 2 groups: 88.1% and 87.8% moisture, 10.9% and 11.1% nitrogen (dry basis) for experimental and control groups, respectively.

In the liver tissue, there also tended to be a higher DNA and a lower PNA content in the case of vit. B_{12}-deficient animals, but these differences were not statistically significant. The calculated amount of PNA per nucleus was less ($P<0.05$) in deficient animals but the difference was so small that it probably has little biological significance. The percentages of tissue nitrogen and moisture did not differ significantly.

Discussion. Liener and Schultze(20) suggested that the high blood urea which they observed in vit. B_{12} deficient newborn rats might result from defective protein anabolism. Perdue and Phillips(21) found high blood urea to be associated with starvation and dehydration in the newborn deficient rat. The possibility that defective carbohydrate metabolism is implicated in the high blood urea observed in the vit. B_{12} deficient rat should not be overlooked. The low liver glycogen observed in this study as well as the poor glucose tolerance observed in adult rats(22) suggests a defect in glycogenesis.

The increased concentration of DNA per gram of brain tissue observed in this study is the result of an increased number of cells per gram of tissue. The content of DNA per nucleus was not changed by a deficiency of vit. B_{12}. This observation confirms the concept that within a species the DNA per nucleus remains constant even when the animal is under stress(23). It appears then that the mass of the average brain cell in the vit. B_{12} deficient offspring is smaller than in the controls. Interpretation of this observation at the cellular level is difficult because of the heterogeneous nature of brain tissue. It may be that all of the brain cells are smaller in mass, or there may be a high proportion of cells with a small mass. An increase in the number of nuclei per gram of brain tissue would result from an increased proportion of glial cells. Globus(24) reported that gliosis was the major causative agent of hydrocephalus in the human cases he observed. Thus, a proliferation of glial cells might furnish a plausible explanation for the closure of the cerebral aqueduct in the hydrocephalic rat brain. The absence of secretory cells in the roof of the cerebral aqueduct has been suggested as the cause of hydrocephalus in the vit. B_{12} deficient rat(5), but the possibility remains that gliosis is a causative factor.

The increased content of DNA and of nuclei per gram of tissue is probably peculiar to brain inasmuch as the differences in liver tissue were not significant. The observation that the PNA per cell decreased in the brain of the deficient animals agrees with the cytochemical observations of Alexander and Backlar(10) that vit. B_{12} administration increased the concentration of PNA in spinal cord and cervical sympathetic ganglia but had no effect on the DNA content of the nuclei. By use of a chemical assay Seigel and Worley (9) observed a 10% lower PNA content in the livers of immature rats from dams fed a vit. B_{12} deficient diet. Liver and pancreas tissue from the deficient rats showed a marked loss of cytoplasmic basophilia. Using growing rats depleted of vit. B_{12} by feeding iodinated casein for a period of 4 weeks, Rose and Schweigert(6) found a lower concentration of both DNA and PNA per gram of liver in the deficient animals but no difference in the concentration per cell. These reports as well as the observations presented here show that a deficiency of vit. B_{12} does not affect the DNA content of the nucleus. There is reasonably good agreement that the PNA concentration in nerve cells is decreased but the effect on liver tissue is not marked.

Summary. Offspring of vit. B_{12} deficient rats usually died within a period of 2 to 3 days, but before death they had a high blood urea and low liver glycogen. At an age of about 1 day, the deoxypentose nucleic acid concentration per gram of brain tissue was higher than normal and this was associated with an increased number of nuclei so that the DNA concentration per nucleus was unchanged. The pentose nucleic acid concentration per cell was lower in brain tissue from deficient offspring, but there was little change in liver tissue.

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Received February 9, 1955. P.S.E.B.M., 1955, v88.

Hydration and Radiosensitivity. (21621)

Roberts Rugh* and Helen Clugston.*
From Marine Biological Laboratory, Woods Hole, Mass., and Radiological Research Laboratory, Columbia University.

Artemia is the brine shrimp which normally lays eggs in brackish water. The egg is provided with a capsule within which development can proceed to the larval stage, which hatches by splitting the shell and emerging. The brackish ponds in which such eggs are laid generally dry up, and the eggs left in great masses on shores. Development within the capsule proceeds to the blastula stage and then, if the capsule is dehydrated, development is suspended until such time as the embryo may again be found in a saline environment. For convenience these blastulae in suspended development will be referred to as "eggs." Such encapsulated eggs survive 15 years or more, then continue development and emerge from their capsules as larvae in 18-24 hours when immersed in salt or sea water at 23-25° C. It must not be presumed, however, that development is always suspended at exactly the same stage of early development, as is attested by the fact that some "eggs" will hatch at 18 and some at 24 hours after hydration has begun. There is a 6 hour disparity in normal hatching time for any batch of "eggs." That increased water content in seeds corresponds directly with increased radiosensitivity has been demonstrated by Caldecott(1). Moss(2) observed lessened radiosensitivity in microorganisms relative to their lessened water content, and Goodrich(3) using grasshopper eggs, found certain stages of development protected and other stages adversely affected by partial dehydration during exposure to x-rays. It is known that x-irradiation alters the water balance(4-5). Artemia "eggs" have been used in research(6) but never in x-irradiation studies.

Materials and method. In the following experiments, exactly 24 and 48 hours after Artemia eggs were placed in sea water, samples of experimental and control batches were removed and immediately fixed in 10% formalin in sea water. The flasks containing eggs were thoroughly shaken to insure homogeneity of the suspension. Four cubic cm of suspension were added to 10 cc of 10% formalin and thoroughly mixed. Counts were made under a dissecting microscope in Syracuse dishes, the bottoms of which had been carefully ruled into 2 mm squares with a diamond point. Two samples were taken from each source, and 2 counts of at least 100 eggs made for each sample. If any of the 4 counts differed by more than 10% from the others, additional counts were taken until this inconsistency was eliminated. All percentage values presented are based upon a total count of at least 400 eggs. Counting was relatively simple because capsules (shells) were not dissolved. Thus, in counting every shell one has an accurate count of the total number of embryos available. Then, in counting those which are hatched one can easily determine the percentage hatching. Motility was simply determined by counting hatched larvae exhibiting lateral appendages which make movement possible. Movement cannot occur without appendages, and in no instance in control cultures were appendages developed without the corollary of motility. X-irradiation was accomplished by alternate parallel tubes run at 184 KVP, 30 MA, at 100 r per second in air. Eggs were placed in a plastic fly box midway between the tubes, and the draft of air from an electric fan was played on the dish to reduce heat conduction from tubes. Actually there was probably no heat factor whatsoever. Even though the output of x-ray source was 100 r/second, there was a considerable lapse of time for x-irradiation to the highest level of 600,000 r. Such an exposure took 1 hour and 40 minutes and was not, therefore, instantaneous. Hydration and development might well have progressed considerably in this lapse of time. Neverthe-

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* Working under contract of the Atomic Energy Commission.

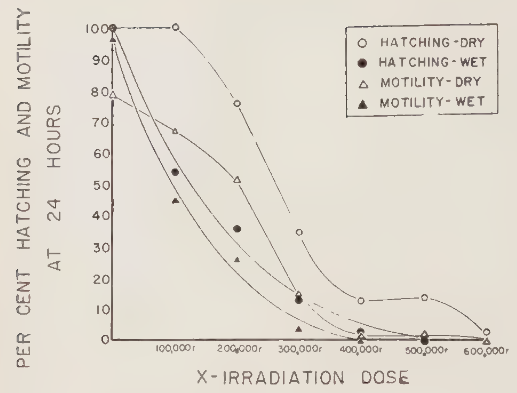

FIG. 1.

less, the following data seem to be of significance.

Results. A. *Radiosensitivity of dry and hydrated artemia eggs as determined by hatching and motility at 24 and at 48 hours.* Artemia eggs were x-irradiated in the dry state and simultaneously others were x-irradiated immediately after placing in filtered sea water. In this wet series, 4 volumes of sea water were added per volume of eggs. X-irradiation at 100 r/second was extended to total doses ranging from 100,000 r to 600,000 r. Following x-irradiation at each level, some eggs were removed and placed in filtered sea water so that the suspension was roughly 0.1%. This allowed adequate hydration for development. Since any delay in hatching would extend the time for some eggs beyond the 24 hour time limit for control eggs, samples were fixed at both 24 and 48 hours. (Fig. 1 and 2.)

Control eggs showed 100% hatching and an almost equivalent (80%) motility value at 24 hours under conditions of temperature and hydration used. Similarly, dry eggs exposed to 100,000 r x-rays and then hydrated were unaffected, but at higher doses there was a decline in hatching percentage following 24 hours in sea water. The 50% hatching value at 24 hours was 260,000 r, but 1.5% hatched at the very high level of 600,000 r. Motility percentages among these eggs were roughly 20% less than hatching percentages.

At 48 hours following hydration of dry-irradiated eggs, hatching percentage had improved so that even after 300,000 r there was 100% hatching, and following 600,000 r, 22% hatching. Thus, x-irradiation caused a definite delay in hatching. This is most obvious if one compares the curves. Since 100,000 r to dry eggs allowed 100% hatching in 24 hours and 300,000 r allowed a similar degree of hatching in 48 hours, *one can say that an increase of 200,000 r caused a delay in hatching time of 24 hours.* This figure can be checked in another manner, namely by determining the dose which will allow 50% to hatch at 24 and 48 hours. These values are 260,000 r and 460,000 r, and the difference again is 200,000 r, although determined at a different level of the curve.

Artemia eggs which were x-irradiated in the wet state (immediately after being placed in sea water) invariably indicated a deleterious effect. At 24 hours, for instance, there was an average reduction in hatching of 20% as compared with eggs irradiated in the dry state. At 48 hours this disparity was even greater (*i.e.*, 30%). Likewise, the 100% lethal dose in wet state was reduced to 500,000 r or more than 100,000 r less than the 100% lethal dose for dry eggs. (Fig. 3).

With regard to motility (Fig. 4) which represents a more advanced stage of development, and which may be a more sensitive indicator of prior damage, the disparity between those x-irradiated in dry and wet states is even greater than when hatching was used as end point. Since hatching and motility never approach the 100% value except in unirradiated controls and at the lowest levels of x-irradiation, we have here two criteria rep-

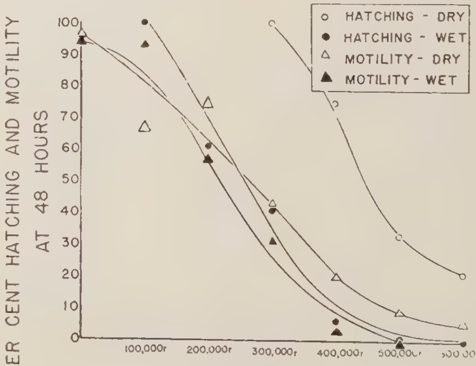

FIG. 2.

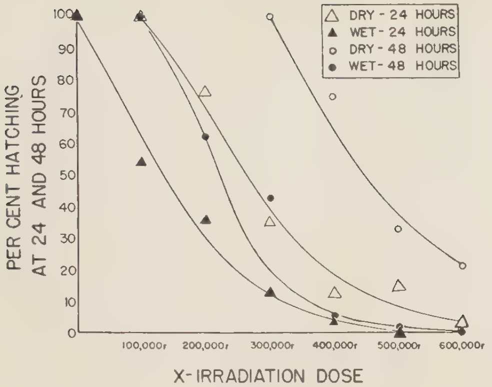

FIG. 3.

resenting 2 stages of development which may be used as indices of radiosensitivity. Further, since at 48 hours the disparity is somewhat greater than at 24 hours, we can say that there is a delay in achievement of motility independent of any effect on hatching.

A further factor in the results following x-irradiation in the wet as opposed to dry state is the approximation of hatching and motility curves (somewhat below the controls) *in the wet state only* (Fig. 1 and 2). In the dry state there is a wide differential between hatching and motility. This suggests that if eggs are x-irradiated in the wet state while development is in progress, there is an alteration of radiosensitivity adversely affecting the mechanisms involved in achieving motility.

Many of the eggs x-irradiated in the dry state are sufficiently damaged to prevent their motility even though not their hatching. The mechanism for hatching must be less radiosensitive than is the mechanism or mechanisms necessary for movement. Hatching and motility represent 2 stages in development, and since motility occurs during a more advanced stage it may be presumed to be a more complicated process than hatching. This is, of course, conjecture and is contrary to the thesis of Bergonie and Tribondeau(7) that radiosensitivity bears roughly an inverse relationship to the stage of development.

Eggs x-irradiated in the wet state do not exhibit such a great disparity between hatching and motility. This suggests that if eggs which are x-irradiated while wet are still able to hatch, they are more apt to be normal and hence motile. However, x-irradiation of eggs in the wet state is more deleterious to either process than is x-irradiation in the dry state. The differential in effect of radiation between hatching and motility processes is greater if eggs are x-irradiated while in the dry state.

At the highest level of x-irradiation studied (*i.e.*, 600,000 r) the difference in hatching results between wet and dry states during exposure is 21% after 48 hours of hydration. The difference in radiosensitivity between wet and dry x-irradiated eggs, as measured by hatching percentage, is much greater at 48 than at 24 hours. This may mean that eggs exposed in a certain stage of development will survive and hatch within the expected time, but those in other, possibly more sensitive stages, will be staggered in their hatching time, and some will be prevented from hatching altogether (Fig. 2). The data on motility show the same relationship, but the disparities are not so great. There is roughly 100,000 r difference between eggs radiated in wet and dry states when one compares motility values at 24 and 48 hours (Fig. 4).

It is necessary to emphasize that x-irradiation at a rate of 6,000 r/min. required such a lapse of time for higher exposures of hydrated eggs that normal metabolic or developmental processes which ensued might well have altered radiosensitivity. Those eggs in the wet state which were exposed to 600,000 r were hydrated for a period of 100 minutes during exposure. This hydration no doubt initiated developmental and metabolic changes within the normal blastula. It would

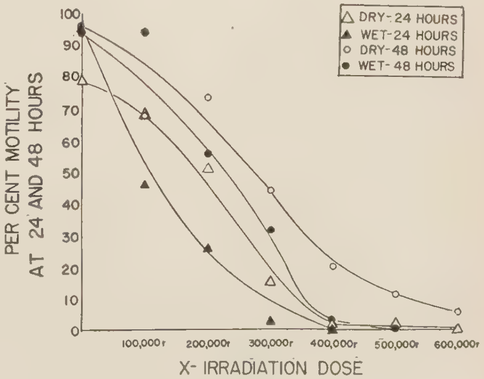

FIG. 4.

be difficult to separate the factor for hydration from that for development in such eggs unless equipment were available for instantaneous x-irradiation at a very high level. Thus we cannot attribute hypersensitivity to surrounding the eggs with an aqueous film. This realization led us to x-irradiate eggs at various intervals after initiation of hydration, and until just before the expected time of hatching of first eggs (*i.e.*, 18 hours).

B. Radiosensitivity of hydrated Artemia eggs at various times before hatching. In preliminary observations it was determined that 250,000 r to dry Artemia eggs would allow approximately 50% to hatch and some 30% to become motile after 48 hours hydration. This level of exposure was then adopted as the standard dose for radiation to be used with eggs at various intervals of hydration, to compare with hatching and motility of controls. Such an exposure required 40 minutes and 40 seconds. This time interval was, therefore, the "margin of error" with respect to an exact state of hydration. When one remembers that there is a disparity of 6 hours in time of hatching in any batch of control eggs similarly hydrated, this suggests that there must be a fair range of variation in development in any group eggs at time of exposure. Nevertheless, it is believed that eggs had passed the blastula stage at the beginning of x-irradiation or hydration. Rate of irradiation was constant, and the data from such a study point up the relative sensitivity of various pre-hatching stages of the Artemia egg following exposure to x-radiations. Since 100% hatching and 98% motility occurred in control eggs by 48 hours after hydration, this was taken as the terminal point of the experiment with which to compare the effects of x-irradiation.

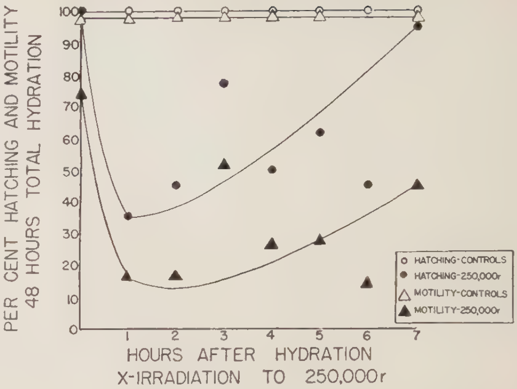

FIG. 5.

The graph (Fig. 5) which represents these data is quite suggestive. Control eggs, similarly hydrated but not x-rayed, showed essentially 100% hatching and motility by 48 hours. Those that were x-irradiated to 250,000 r showed maximum radiosensitivity (expressed as failure to hatch) when x-irradiation was done 1 hour following hydration. In these there was 36% hatching. Hatching percentages fluctuated among eggs irradiated at intervals following hydration but those which had been hydrated 7 hours post irradiation showed a definite increase in radioresistance over those hydrated only 1 hour. This may be explained as follows: 1) Hydration encourages or allows the egg to proceed in its development immediately, and this sudden revivification might well be the cause for the heightened radiosensitivity, in a manner similar to that associated with accelerated metabolism. 2) X-irradiation might conceivably increase permeability of the egg capsule and contained blastula to sea water. Such an increase in permeability at a time when hydration is normally occurring might well adversely affect the embryo. 3) There is experimental support for the thesis that the blastula stage is most radiosensitive of all developmental stages of an embryo(8). The dry Artemia egg is a blastula in a suspended state of development. Since development of the controls proceeds from the blastula to fully formed and motile larva in 48 hours after hydration at temperatures used, development is normally quite rapid. The data on motility of embryos x-irradiated at different intervals of hydration are shown in Fig. 5. Following an initial drop to 17% in those x-irradiated 1 hour following hydration, there was a distinct rise in motility percentages to termination of observation period at 7 hours. The general trend of the curve suggests that there is an initial post-hydration radiosensitivity followed by increasing radioresistance as development proceeds. This may reflect progressive radioresistance of the embryo

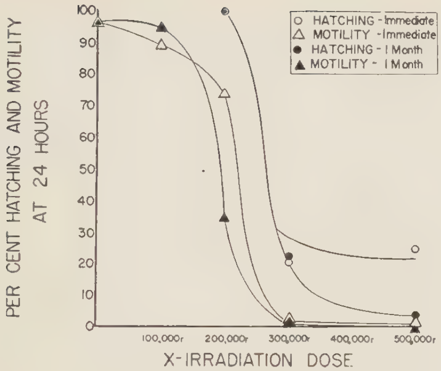

FIG. 6.

with its development as first suggested by Bergonie and Tribondeau (Fig. 5).

C. Effect of delayed hydration following x-irradiation of dry eggs. Dry eggs were x-irradiated to various levels from 100,000 r to 500,000 r and eggs from each dose level separated into two batches. One batch was hydrated immediately and counts of hatching and motility made at 24 and at 48 hours. The other batch from each dose level was set aside at room temperature (about 23-25°C) for 1 month and then hydrated in sea water. It was thought that possibly a delay in hydration following x-irradiation might allow for some degree of recovery of embryos from irradiation damage. Curves of hatching and motility (Fig. 6, 7) for the 2 batches proved remarkably alike, particularly when comparisons were made of eggs hydrated for 24 hours. Those that had been hydrated for the longer period of 48 hours showed a slight difference in curves, indicating greater damage in eggs which had had a month's delay in hydration. Thus, there was no "recovery" but rather a more marked effect when hydration was delayed one month. Data are presented in terms of hatching and motility at 24 and at 48 hours, comparing eggs x-irradiated and hydrated immediately with those x-irradiated and kept dry for one month (Fig. 7). Hydration of these sets of eggs was simultaneous, and under identical conditions of light, temperature, and dilution.

Delay in hydrating x-irradiated eggs caused a reduction of 21% in hatching at the very high dose of 500,000 r, while motility showed a 39% reduction at the lower dose of 200,000 r. The value for motility approached zero at 300,000 r and above, both for immediate and for 1 month series.

Hydration for 48 hours gave slightly different values. Hatching disparity between eggs hydrated immediately and with 1 month delay was 31% at 300,000 r and 18% at 500,000 r. The latter figure is so close to zero that the disparity value may not be accurate. The disparity in motility data between eggs hydrated immediately and those hydrated after a 1 month delay showed very little difference at 200,000 r, but at 300,000 r they differed by 54%. Both approached zero at 500,000 r. Thus maximum disparity was at 300,000 r following 48 hours of hydration. In every comparison, however, the delay in hydration for 1 month was detrimental to either hatching or motility values.

Summary and conclusions. 1. Artemia "eggs," essentially in the blastula stage in suspended development, can tolerate about 200,000 r more x-radiation in the dry than in the wet state, if criteria of hatching and motility after 48 hours hydration are considered. 2. The disparity between hatching and motility curves is greater in dry than in hydrated eggs following x-irradiation. Requirements for hatching must be less radiosensitive than those for motility, since there was always a disparity between these 2 steps in development. It is probable that hydrated eggs are so radiosensitive that only the hardiest survive, and these reach motility. 3. Hydrated

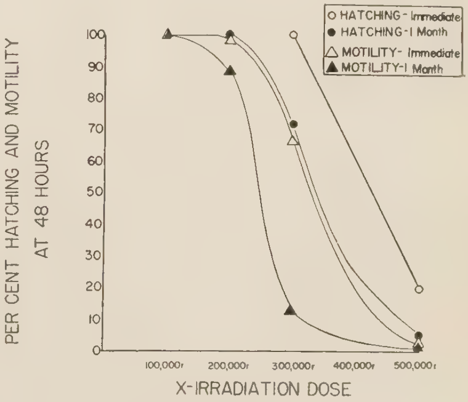

FIG. 7.

eggs are variously radiosensitive, depending upon duration of hydration prior to x-irradiation. There is an immediate drop in radioresistance following hydration, with gradual recovery of this resistance so that after 7 hours of hydration eggs are quite radioresistant again. 4. Delay of one month between x-irradiation and hydration was detrimental to eggs, resulting in reduced values for hatching and motility. The greatest disparity was at 300,000 r.

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Received February 11, 1955. P.S.E.B.M., 1955, v88.

Diffusion of Fluids in Skin after Exposure to X-Irradiation.* (21622)

G. PRODI AND R. MICELI. (Introduced by Giovanni Favilli.)

From Center of Bologna for Cancer Research, Divisions of Radiology and of Experimental Oncology.†

This paper reports the results of investigations concerning eventual alterations induced by X-rays on the non-cellular structures of connective tissues, with special attention to the constituents of the ground substance. We proposed to make use of the so-called diffusion test, by injecting either India ink or diphtheria toxin into skin tissues previously exposed to irradiation. It is well known that the mode and time of diffusion in the connective tissues are, within some limits, indicative of both qualitative and quantitative characteristics of the ground substance. The present investigations aimed at studying the diffusion phenomenon at few minutes following irradiation.

Material and method. Experiments were carried out on adult albino rabbits averaging 2 kg in body weight. For irradiations a contact therapy apparatus was used (5 cm foc. dist; 50 Kw; 5 mA; 0.5 AL filter). Each rabbit's flanks, carefully shaved, were divided into 2 or 3 square areas. The right side areas were subjected to irradiation; the left side used as controls. A few minutes after irradiation each square area of both right and left flanks was intradermally injected with 0.3 ml of India ink‡: a swelling was thus obtained of 1 cm diameter, bulging out 4-5 mm from the skin surface. The diffusion areas, represented by well evident black spots on the skin, were measured 30 minutes, 1 hour, 3 hours after injection, by drawing the outline of the spots on cellophane stretched on the skin and transferring it on coordinate paper so as to obtain the spotted surface in mm². Table I reports data of the spotted area 3 hours after inoculation. Other experiments were made using diphtheria toxin as diffusion indicator in place of India ink. Irradiation followed immediately and the control areas were intradermally injected with

TABLE I. Diffusion of India Ink (in mm²) 3 Hr after Injection.

Rabbit	Dose, r	Side Control	 Irradiated	△
1	250	609	686	77
	500	577	690	113
	750	503	793	290
2	250	520	495	–25
	500	505	660	155
	750	560	655	95
3	1000	441	809	368
	2000	512	986	474
4	1000	680	1100	420
	2000	570	1050	480
5	1000	480	810	330
	2000	460	1200	740
6	1000	430	700	270
	2000	450	880	430

△—difference between diffusion areas on control and irradiated sides.

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* This work supported by a grant from the Damon Runyon Memorial Fund for Cancer Research.

† Address: Via S. Giacomo 14, Bologna, Italy.

‡ "Pelikan" India ink of Gunther Wagner Co.

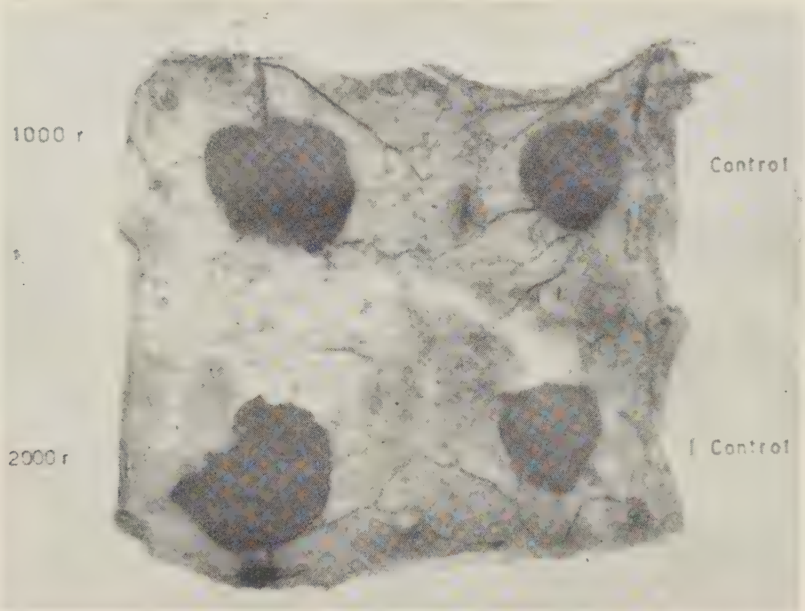

FIG. 1. Rabbit 6 (Table I) inoculated with India ink. 3 hr later the animal was sacrificed, skin removed, pinned on board and dried. Photograph taken on subcutaneous side.

0.3 ml of a solution containing an amount of toxin equal to 6 doses used for the reaction of Shick: which means that the content of an ampoule for 10 Shick's doses of dry toxin was mixed with 0.5 ml of saline, and 0.3 ml of this mixture were inoculated.§ Diffusion areas of toxin in the skin do not appear before 24 hours following inoculation; they resemble erythematous patches, followed within 36-72 hours by sharply outlined necrosis. The surface areas were measured after 24-36-48 hours with the same technic as the India ink diffusion areas. Table II shows data 36 hours after inoculation.

Experiments with India ink. Two rabbits were irradiated on the 3 square areas of the right flank with 250, 500, 750 r each. The left flank corresponding areas were used as controls. Four rabbits were given 1000 and 2000 r on the 2 square areas of the right flank. The corresponding areas on left flank were used as controls. Results are shown in Table I; Fig. 1 shows rabbit 6. Throughout such experiments diffusion with India ink in the skin previously exposed to irradiation was considerably wider than diffusion in unirradiated skin, with the exception of the square areas where the minimum dose of 250 r had been employed, which proved to be insufficient to produce a steady significant increase of diffusion. On the contrary, considerable increase, as compared with controls, is seen on areas irradiated with 500 r. The higher the doses used, the greater the increase of diffusion.

Diphtheria toxin. Two rabbits were irradiated on the 2 square areas of the right flank with 1000 and 2000 r each, (Table II and Fig. 2). These experiments fully confirm that diffusion in irradiated skin was wider than in normal skin. The above data give evidence of an increased diffusion of the inoculated substances in irradiated *vs* un-

TABLE II. Diffusion of Diphtheria Toxin (in mm²) 36 Hr after Injection.

Rabbit	Dose, r	Side Control	 Irradiated	Δ
1	1000	410	590	180
	2000	340	580	240
2	1000	380	810	430
	2000	350	920	570

Δ—difference between diffusion areas on control and irradiated sides.

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§ Diphtheria toxin prepared by the Istituto Sieroterapico e Vaccinogeno Sclavo of Siena.

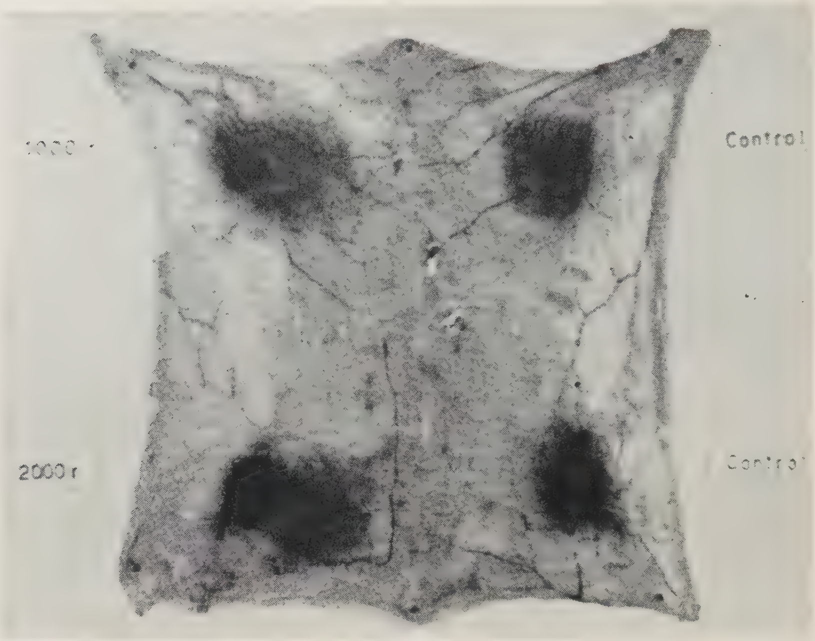

FIG. 2. Rabbit 2 (Table II) inoculated with diphtheria toxin. 48 hr later the animal was sacrificed, skin removed, pinned on board and dried. Photograph taken on external side.

irradiated skin. The same phenomenon is elicited when hyaluronidase is injected in normal skin together with India ink or diphtheria toxin. The immediate alterations of cutaneous and subcutaneous tissues after irradiation are commonly referred to phenomena of predominantly vascular nature, but they are not sufficient to explain the increase of diffusion. There is considerable evidence showing the relationship between diffusion in connective tissues and the chemical and physical state of the mucopolysaccharide fractions, especially hyaluronic acid, which constitute the ground substance. It seems quite possible, from our experiments, that Roentgen irradiation by depolymerizing such constituents *in vivo,* should induce an increase of diffusion similar to the increase induced by hyaluronidase. This interpretation is supported by observations made on the axolotl (Prodi and Miceli(1)), whose cutaneous and subcutaneous tissues, fairly rich in highly metachromatic substance, lose this character after irradiation. Similar observations were made by Pisani and Romanini(2) on human tumours and by Verga and Cali(3) on the umbilical cord. Further support to these considerations is given by an observation of Schoenberg *et al.*(4) on the fall of viscosity of hyaluronic acid after irradiation. It cannot be excluded that capillary lesions produced by irradiation (Neumayr and Thurner(5)) may play some rôle in the phenomenon observed, by changing the water content of the connective tissue.

Recently Upton and Gude(6) observed an increase of diffusion of Evans Blue intradermally injected in rats subjected to total irradiation, when the dye was inoculated 4-7 days after irradiation. It is doubtful, however, whether the increase of diffusion observed in these experiments is to be referred to the same mechanism as the increase of diffusion observed in our experiments. This change could be the indirect result of irradiation through nervous and hormonal mechanisms which control the production of the ground substance or it could be caused by capillary lesions. Also Kisselev(7) noted an increase in diffusion of a dye injected into the rabbits' dermis after having subjected it to X-irradiation. In the short summary of Kisselev's paper available to us there is no mention of the time elapsed between irradiation and diffusion test.

Summary. India ink and diphtheria toxin were injected intradermally in the rabbit, in previously X-irradiated and unirradiated skin areas, the latter being symmetrically opposite to the former. In the irradiated areas the spreading surface of the injected materials was larger than in control areas. It is assumed that the increase of diffusion is due to depolymerization of the mucopolysaccharides of the ground substance by X-irradiation.

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Received February 14, 1955. P.S.E.B.M., 1955, v88.

Effect of Chlorpromazine on Survival Time in Irradiated Mice.* (21623)

Thomas J. Haley, W. G. McCormick, and Eve F. McCulloh.

From Division of Pharmacology and Toxicology, Atomic Energy Project, School of Medicine, University of California at Los Angeles.

Chinn and Sheldon(1) showed that chlorpromazine in doses of 5 to 10 mg/kg increased survival time in x-irradiated dogs. They did not see any beneficial effect in mice and guinea pigs but they stated that, "the apparent increase in survival time after chlorpromazine requires confirmation with larger series of animals." We have studied the protective effect of chlorpromazine in mice and have observed that this drug does increase mean survival time in mice at the 5 mg/kg dose level. However, larger doses were detrimental to the irradiated mouse.

Methods. Male, CF-1 strain mice, weighing an average of 25 g each, were arranged in groups of 20 animals each and given single, intraperitoneal injections of chlorpromazine according to the schedule shown in Table I. Except during irradiation, the animals were maintained in an air-conditioned room at 72 ± 5° F and were fed a diet of Rockland pellets supplemented weekly with additional vit. A and D. The 550 r radiation dose was administered from above and below the mice with two 250 KVP Picker Industrial Units operating simultaneously. The technical factors were: 250 KVP; 15 ma; FOD 100 cm; filters, 0.21 mm Cu inherent, 0.5 mm Cu parabolic and 1.0 mm Al; HVL 2.02; size of field—total body; r/min measured in air 17.27. Both units were calibrated before and after each experiment with a Victoreen thimble r-meter. The animals were restrained in a plastic cage similar to the one described previously for guinea pigs(2). The results obtained were analyzed statistically by the Litchfield method(3).

Results. The results obtained were given in Table I. The saline control animals responded in the same manner as has been reported from this laboratory for the last several years(4-7).

The control chlorpromazine groups showed the usual increased toxicity for increased dosage, but it appeared that the drug was more toxic via the intraperitoneal route than reported for the intravenous route by Courvoisier *et al.*(8). Central depression was more marked at the higher doses and it was observed that a preliminary test group which received 40 mg/kg was sedated for at least 24

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* This article was based on work performed under contract between the Atomic Energy Commission and the University of California at Los Angeles.

TABLE I. Effect of Chlorpromazine in Irradiated Mice.

Group	Medication days	Dose	ST_{50}* and range in days	Slope and range	Total mortality	
					Day	%
Rad† Saline	Post-R 1‡	0.5 cc	9.9 (9.0-10.8)	1.23 (1.16-1.31)	14	100
Non-rad Chl†	—	— 5 mg/kg	—	—	—	—
Rad Chl	Pre-R§	5	16.7 (13.5-20.7)	1.63 (1.38-1.93)	28	85
Rad Chl	Post-R 1	5	9.6 (7.4-12.4)	1.81 (1.51-2.17)	21	100
Rad Chl	" 7	5	9.9 (8.6-11.4)	1.39 (1.26-1.54)	19	100
Non-rad Chl	—	— 10 mg/kg	—	—	6	15
Rad Chl	Pre-R	10	4.8 (4.3- 5.4)	1.33 (1.20-1.47)	11	100
Rad Chl	Post-R 1	10	6.6 (5.2- 8.5)	1.76 (1.48-2.09)	14	100
Rad Chl	" 7	10	8.6 (7.8- 9.5)	1.25 (1.16-1.34)	11	100
Non-rad Chl	—	20	6.2 (4.7- 8.3)	1.85 (1.45-2.36)	9	65
Rad Chl	Pre-R	20	2.1 (1.6- 2.8)	1.91 (1.56-2.33)	5	95
Rad Chl	Post-R 1	20	4.0 (3.6- 4.4)	1.26 (1.17-1.36)	7	100
Rad Chl	" 7	20	8.8 (8.0- 9.7)	1.24 (1.16-1.33)	11	100

* ST_{50} = Day on which 50% of animals alive. All values at $P = 0.05$.
† Rad = Radiated; Chl = Chlorpromazine.
‡ Post-R = Post-irradiation.
§ Pre-R = Pre-irradiation. Injected one-half hr pre-irradiation.

hours. This sedative effect differed from that seen with barbiturate anesthesia, in that respiration appeared normal rather than depressed.

The 5 mg/kg dose of chlorpromazine given prior to irradiation was definitely beneficial, increasing the ST_{50} day by almost 50%. It also increased total survival by 15%. Administration of this dose of drug was neither beneficial nor detrimental when given on post-irradiation days 1 or 7. On the other hand, the higher doses before or after irradiation acted synergistically with the irradiation, decreasing both the ST_{50} and total mortality days. However, this synergism was less on post-irradiation day 7. The exact mechanism whereby chlorpromazine exerts its beneficial effect is obscure because the drug has so many diverse actions in the animal organism (9). We are certain, however, that hypothermia and artificial hibernation with chilling and a chlorpromazine-adrenergic blocker mixture similar to that of Laborit *et al.*(10) are not involved because an experiment of this type was highly detrimental to mice(11). Furthermore, injections of Hydergine (equimixture of dihydroergocornine, dihydroergocristine, and dihydroergokryptine), 5% glucose and chilling for one-half hour prior to irradiation decreased survival time.

Conclusion. Pre-medication with 5 mg/kg of chlorpromazine intraperitoneally significantly increased the mean survival time in irradiated mice. Post-irradiation medication with this dose of drug was neither beneficial nor detrimental but doses of 10 and 20 mg/kg decreased survival time by acting synergistically with the irradiation. The sensitivity of the irradiated animals to medication was discussed.

The authors wish to thank Smith, Kline and French Laboratories for the chlorpromazine used.

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Received February 15, 1955. P.S.E.B.M., 1955, v88.

Serum Liproteins in Experimental Diabetes. IV. Effect of Prolactin.* (21624)

GIORGIO GALANSINO,† ANGELO FASOLI,‡ ALFRED M. MAGILL, AND PIERO P. FOÀ.
From Department of Physiology and Pharmacology, Chicago Medical School, Chicago 12, Ill.

Previous studies from this laboratory have revealed that the serum lipoprotein pattern of dogs differs from that of man and is modified by pancreatectomy and insulin(1), by heparin(2) and by anterior pituitary growth (diabetogenic) hormone(3). Since, under certain experimental conditions, prolactin causes hyperglycemia(4,5), a study of the effects of this hormone on serum lipoproteins of normal and depancreatized dogs was undertaken.

Materials and methods. Eleven normal and 5 depancreatized mongrel dogs of both sexes were used in these experiments. All dogs were kept on a constant diet of commercial dog food and were fasted for about 18 hours before the experiment. Depancreatized dogs were treated as previously described(6) and received supplements of raw pancreas and pancreas powder.§ After securing a control sample of venous blood, purified prolactin (Armour Lot R-491154)‖ was injected intravenously (20 I. U./kg in 0.9% saline). Blood samples were again obtained 30 and 120 minutes after the injection, at a time when the effect of prolactin on blood sugar was most pronounced. Eight of the 11 normal dogs received 2 or 3 additional intravenous injections of prolactin at alternate days and then the experiment was repeated. Four control experiments were performed using an amount of ACTH (0.4 mg/kg) similar to that contained in the prolactin preparation used. Blood glucose was determined in duplicate according to Nelson(7). Serum aliquots were studied by means of electrophoresis on filter-paper and the cholesterol and lipid phosphorus content of the serum and of the electrophoretic fractions was determined as described in the first paper of this series(1). The statistical significance of the results was calculated with the method of Fisher(8).

Results. (Table I). The results confirm the following observations previously reported: 1. In the dog, most of the serum

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* These studies were aided by contract between Office of Naval Research, Department of the Navy and Chicago Medical School, and by grant from the U.S.P.H.S.

† International Exchange Fellow. Present address: Istituto di Anatomia Patologica, Università di Milano, Italy.

‡ Present address: Clinica Medica, Università di Milano, Italy.

§ Gift of Dr. N. Ercoli of Armour Laboratories.

‖ Gift of Drs. I. Bunding and R. S. Steelman of Armour Laboratories.

TABLE I. Effect of Prolactin on Serum Cholesterol and Lipid Phosphorus Fractions in Normal and Depancreatized Dogs. (Average values.)

Min. after inj.	Cholesterol						Lipid phosphorus						Blood glucose, mg %
	mg/100 cc serum												
	A_{α_1}	α_2	β_1	$\beta_2\gamma$	Sum of fractions	Total*	A_{α_1}	α_2	β_1	$\beta_2\gamma$	Sum of fractions	Total*	
						Prolactin—first inj.							
0	142	15	25	10	192	211	6.0	.6	.4	.1	7.1	7.9	90
30	177	14	20	6	217	201	6.2	.6	.3	.0	7.1	7.8	75
120	171	10	17	7	205	216	6.4	.5	.4	.0	7.3	8.1	67
						Prolactin—subsequent inj.							
0	139	16	20	9	184	205	7.1	.6	.6	.2	8.5	9.2	77
30	142	9	15	6	172	190	6.7	.9	.4	.0	8.0	9.1	98
120	150	18	24	10	202	208	7.4	.9	.2	.4	8.9	9.5	77
						Prolactin—diabetic dogs							
0	259	26	17	4	306	320	6.4	1.0	1.2	.6	9.2	8.8	222
30	270	24	20	2	316	320	7.2	1.4	.6	.4	9.6	10.8	266
120	270	27	20	2	319	312	7.0	1.4	.8	.4	9.6	10.4	278
						ACTH—first inj.							
0	175	10	12	5	202	200	9.6	.2	.8	.4	11.0	12.1	75
30	138	10	18	4	170	194	9.6	.4	.4	.6	11.0	12.6	74
120	156	7	10	5	178	194	9.0	.4	.5	1.1	11.0	12.6	77
						ACTH—subsequent inj.							
0	160	12	10	3	185	200	6.5	.4	.6	.3	7.8	8.5	77
30	172	6	17	3	198	200	7.5	1.2	.3	.0	9.0	8.7	75
120	159	15	8	6	188	198	7.4	1.2	.5	.1	9.2	10.0	80

* By direct determination.

lipids are carried in the albumin-α-globulin fractions and only a small amount in the β-globulins. This is different from the situation in man(1,9). 2. The serum of depancreatized dogs under insulin control is hyperlipemic and has an elevated $\frac{\text{cholesterol}}{\text{lipid P}}$ ratio(1). A comparison of the cholesterol and lipid P values obtained before and after single or repeated injections of prolactin in normal or diabetic dogs fails to reveal statistically significant changes in spite of the fact that the first injection of prolactin in the normal dog causes a marked hypoglycemia and repeated injections in normal and diabetic dogs cause marked hyperglycemia. Control experiments with ACTH also gave negative results.¶

Discussion. Prolactin does not modify serum cholesterol, lipid phosphorus and lipoprotein pattern of normal and depancreatized dogs, as it does not modify the fat content of liver and carcass of normal male rats(10). Prolactin, however, causes marked changes in blood sugar concentration(5), confirming the hypothesis that the changes in serum lipoproteins observed after pancreatectomy, insulin and the administration of growth hormone are not exclusively related to the changes in blood sugar, but may be the results of a direct action on lipid transport and metabolism (11,12). The ACTH experiments were designed as controls for the prolactin experiments. In view of the small doses of ACTH used, no conclusions can be reached regarding the effects of this hormone on serum lipoproteins.

Conclusion. Single or repeated injections of prolactin do not cause significant changes in serum cholesterol, lipid phosphorus and lipoprotein pattern of normal and depancreatized dogs.

¶ $P > 0.05$ in all cases.

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Received February 18, 1955. P.S.E.B.M., 1955, v88.

Ingestion and Destruction of Influenza Virus by Free Living Ciliate *Tetrahymena pyriformis*.* (21625)

VINCENT GROUPÉ, ERNEST C. HERRMANN, JR., AND FRANK J. RAUSCHER.

From Institute of Microbiology, Rutgers University, State University of New Jersey, New Brunswick.

A previous report from this laboratory(1) showed that when influenza B virus was added to axenic (bacteria-free) cultures of *Tetrahymena pyriformis* (*T. geleii*) the virus was affected in at least 2 ways: First, the infective titer was substantially reduced by a factor present in both killed and viable cultures, and second, the viral hemagglutinin was inactivated or destroyed by actively multiplying but not by mature protozoal cultures. The former phenomenon recalls to mind the fact that anti-bacterial lipids have been obtained from similar cultures of *T. pyriformis* (2). The latter phenomenon suggests that *T. pyriformis* might feed on influenza virus, since it is well known that *T. pyriformis* and certain other ciliates feed upon bacteria. The studies reported here support this suggestion.

Methods. The PR-8 strain of influenza A virus, the Lee strain of influenza B virus, and the California strain of Newcastle disease virus (NDV) were propagated in the allantoic sac of chicken embryos. Viral hemagglutinin(3,4) and complement fixing(5) titers were determined in the usual manner. End points were determined in both tests by serial 2-fold dilution to extinction of each sample. Optical density was used to measure protozoal multiplication as described by Kidder and Dewey(6). Two sublines of *T. pyriformis* were maintained by weekly transfer in an (a) allantoic fluid containing influenza B virus and (b) PPL medium (2% proteose peptone and 0.5% liver extract in distilled water), respectively. *Glaucoma scintillans* was maintained in axenic cultures in a medium containing 2% proteose peptone, 0.1% glucose, 0.1% liver extract, and 0.5% yeast extract in distilled water. *Euglena gracilis* and *Astasia klebsii* were maintained in a medium containing 0.5% tryptone (Difco), 0.2% $NaC_2H_3O_2$, 0.15% KH_2PO_4, 0.5%

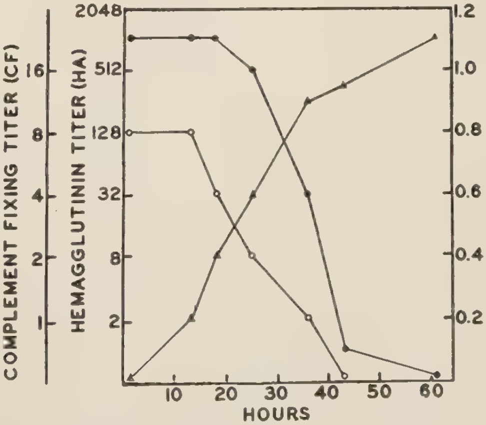

FIG. 1. Inactivation of influenza B viral hemagglutinin and complement fixing antigen by *T. pyriformis*. ▲ = OD; ● = HA; ○ = CF.

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* This investigation supported by a research grant from the National Microbiological Institute of National Institutes of Health, Public Health Service, and by funds provided by Rutgers Research and Endowment Foundation.

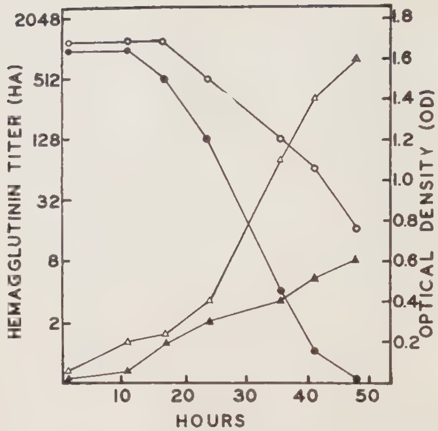

FIG. 2. Effect of additional nutrients on inactivation of influenza B viral hemagglutinin by *T. pyriformis*. ● = HA without added nutrients; ○ = HA with added nutrients; ▲ = OD without added nutrients; △ = OD with added nutrients.

KNO_3, 0.1% $MgSO_4 \cdot 7H_2O$, and 0.01% NaCl in distilled water. *Experimental design.* Pools of allantoic fluids were stored at –10°C and aliquots were thawed and clarified by centrifugation before use. In each experiment 40-90 ml amounts of allantoic fluid containing 200 units each of penicillin and streptomycin per ml were placed in 250 ml Erlenmyer flasks. Each flask was inoculated with 1-2 ml of a 24-48 hour old culture of protozoa or saline and incubated at 28°C. Three to 5 ml aliquots were removed from each flask at appropriate intervals, the optical density was recorded, and formalin was added to 0.05% to kill the protozoa. Each aliquot was then clarified by centrifugation and stored at 5°C for assay of its hemagglutinin and complement fixing activity later that week.

Results. Axenic cultures of *T. pyriformis* that grew luxuriantly in PPL medium also grew well and could be maintained in serial culture in allantoic fluid collected from chicken embryos infected with influenza B virus. Growth was poor in allantoic fluid collected from normal embryos. The data presented in Fig. 1 show the effect of multiplication of *T. pyriformis* in allantoic fluid on its viral hemagglutinin and complement fixing antigen content. The details of procedure are described above under "Methods." It will be seen that the rapid increase in the protozoal population was accompanied by a corresponding rapid decrease in both viral hemagglutinin and complement fixing antigen. All attempts to demonstrate production of an inhibitor of viral hemagglutination *in vitro* by *T. pyriformis* in PPL medium, or to demonstrate absorption of viral hemagglutinin by concentrated protozoa failed. It will be recalled that the complement fixing antigen of influenza virus was found to be the most stable property of the virus when exposed to ultraviolet light(7) or nitrogen mustard(8). It appears, therefore, that *T. pyriformis* is capable of completely destroying influenza B virus.

Data presented in Fig. 2 show the effect of added nutrients on protozoal growth and reduction of viral hemagglutinin in allantoic fluid infected with influenza B virus. In this experiment 30 ml of a sterile aqueous solution of 5% proteose peptone, 1% liver extract, and 1% yeast extract was added to an equal volume of allantoic fluid. Otherwise, this experiment was identical in procedure to that described above. It will be seen that the presence of additional nutrients in allantoic fluid affected the culture in 2 ways: (a) Protozoal growth was markedly increased, and (b) reduction of hemagglutinin was delayed but not substantially decreased.

Data presented in Table I show that it was possible to recover influenza B viral hemagglutinin from frozen and thawed *T. pyriformis*. In this experiment, as before, aliquots of a culture of *T. pyriformis* were collected at the intervals indicated and formalinized to kill the protozoa. In this case, however, the protozoa were collected by centrifugation, resuspended in saline to the original

TABLE I. Recovery of Hemagglutinin from *T. pyriformis*.

	Allantoic fluid				
	Hr of incubation at 28°C				
Tetrahymena pyriformis	0	10	14	34	38
Control:					
No protozoa	512*	512	512	512	512
Protozoal culture:					
Supernatant fluid	512	512	512	128	32
Sediment (protozoa) frozen and thawed concentrate		32	16	16	16

* Reciprocal of hemagglutinin titer.

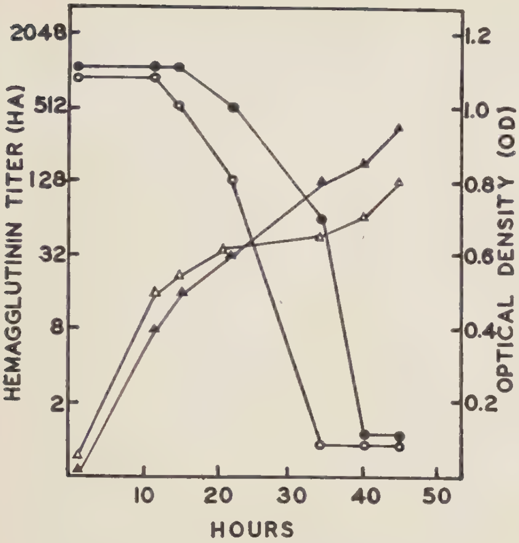

FIG. 3. Inactivation of hemagglutinins of influenza A and Newcastle disease virus (NDV) by *T. pyriformis*. ● = HA of influenza A virus; ○ = HA of NDV; ▲ = OD with influenza A virus; △ = OD with NDV.

volume, again sedimented by centrifugation, and then resuspended in a volume of saline equal to ¼ of the original volume of the sample. The washed, 4X concentrated protozoal suspension was then frozen and thawed 3 times, clarified by centrifugation, and tested for the presence of viral hemagglutinin. The data show that a small, but detectable amount of hemagglutinin was recoverable from the protozoa throughout the period of decline in titer of hemagglutinin in allantoic fluid. Similarly prepared concentrates of the ciliate grown in the absence of virus failed to agglutinate red blood cells.

Thirty-three consecutive subinoculations of *T. pyriformis* in allantoic fluid infected with influenza B virus failed to enhance the ability of the protozoa to reduce viral hemagglutinin. Thus, when the two sublines of *T. pyriformis,* maintained on allantoic fluid and PPL medium, respectively, were compared, the rates of protozoal growth and of reduction in viral hemagglutinin were almost identical. Further, exposure of allantoic fluid infected with influenza B virus to a temperature of 50°C for 1 hour also failed to alter the rate of growth of the protozoa or the reduction in hemagglutinin.

Data presented in Fig. 3 show that *T. pyriformis* also multiplied in allantoic fluid collected from eggs infected with influenza A virus or Newcastle disease virus (NDV) and that a corresponding reduction of viral hemagglutinin occurred in each instance, comparable with that obtained with influenza B virus. The procedures employed in these experiments were identical with those described above under Methods.

In Table II data are presented showing that axenic cultures of *Glaucoma scintillans, Euglena gracilis,* and *Astasia klebsii* all failed to reduce viral hemagglutinin whereas *T. pyriformis* effectively reduced hemagglutinin titer under comparable conditions.

Discussion. The data presented dealing with the multiplication of *T. pyriformis* in allantoic fluid collected from eggs infected with influenza B virus clearly point to the conclusion that the protozoa ingest the virus particles and destroy them. The evidence for this conclusion may be summarized as follows: (a) Hemagglutinin and complement fixing antigen were inactivated at approximately the same rates. (b) Inactivation of virus was inversely related to the increase in the protozoal population. (c) Viral hemagglutinin was recoverable in low concentration from washed protozoa. (d) The presence of additional nutrients delayed but did not prevent reduction of the viral hemagglutinin content of the medium by *T. pyriformis*. Failure of the flagellates *Euglena* and *Astasia* to remove virus is understandable since those protozoa do not possess oral parts. However, failure of the ciliate *Glaucoma,* which is morphologically similar to *Tetrahymena,* to affect virus may be due to failure to ingest virus, but it is equally possible that *Glaucoma* is unable to digest virus and therefore rapidly excretes the virus without alteration of its hemagglutinin.

The fact that *Tetrahymena* is capable of ingesting and destroying influenza virus suggests that free living ciliates may play a role in the removal of certain viruses in sewage disposal plants and conceivably in soils and streams. It is also conceivable that certain viruses may prove to be useful as a nutrient in initiating axenic cultures of other ciliates.

TABLE II. Failure of Certain Protozoa to Inactivate Influenza B Viral Hemagglutinin.

	Duration of incubation at 28°C					
	0 hr		48 hr		7 days	
Protozoan	HA	Growth	HA	Growth	HA	Growth
Ciliates:						
Tetrahymena pyriformis	128	—	2	++	0	+++
Control	128	—	128	—	128	—
Glaucoma scintillans	64	—	64	+	64	+++
Control	64	—	64	—	64	—
Flagellates:						
Astasia klebsii	64	—	64	++	128	+++
Control	64	—	64	—	64	—
Euglena gracilis	64	—	32	++	64	+++
Control	64	—	64	—	64	—

HA = Reciprocal of hemagglutinin titer.
Amount of protozoal growth: +, slight; ++, moderate; +++, luxuriant.

Summary. The hemagglutinin and complement fixing antigen of influenza B virus were inactivated at approximately the same rate by *T. pyriformis.* Inactivation of virus was inversely related to the increase in protozoal population. Hemagglutinin was recoverable in low concentration from washed protozoa. The presence of additional nutrients delayed but did not prevent reduction of the viral hemagglutinin content. The hemagglutinins of influenza A virus and of Newcastle disease virus were also inactivated by actively growing cultures of *T. pyriformis.* The flagellates *Euglena gracilis* and *Astasia klebsii* and the ciliate *Glaucoma scintillans* failed to affect influenza B viral hemagglutinin.

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Received February 18, 1955. P.S.E.B.M., 1955, v88.

Effect of Tocopherol on Carotene Conversion.[*] (21626)

J. G. BIERI.

From Department of Biochemistry and Nutrition, University of Texas Medical Branch, Galveston.

It is known that small supplements of α-tocopherol enhance the utilization of low doses of β-carotene while larger amounts diminish the effectiveness of the pro-vitamin (see Hebert and Morgan(1) for a recent review). Although this adverse effect of α-tocopherol has been presumed to be on the stability, and possibly on the absorption, of carotene in the intestine, High, Woods and Wilson(2) in noting a similar occurrence with other antioxidants (alkyl hydroquinones) suggested that these substances, as well as tocopherol, may interfere directly in the enzymatic conversion of carotene to vit. A.

By injecting carotene intravenously(3), it is possible to circumvent the factors of destruction and absorption in the intestine. If tocopherol does affect the enzymatic oxidation

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[*] These studies were aided by contract between Office of Naval Research, Department of Navy, and the University of Texas Medical Branch.

of carotene to vit. A, it might be expected that utilization of injected carotene would be altered by increased levels of tissue tocopherol.

Methods. Weanling, male Holtzman rats caged singly were depleted of vit. A by feeding a synthetic, A-free diet(4) not containing added tocopherol. The 2% cottonseed oil in this ration would supply about 0.18 mg total tocopherols/10 g ration, calculated from the analyses of Harris, *et al.*(5). On the 11th day, the rats were grouped by weight and from the 12th to 16th day, inclusive, 0.2 ml of an aqueous solution of 20% Tween 40 containing 0, 4, 10 or 20 mg α-tocopherol (Merck) was given daily by stomach tube. On the 18th day, when about half of the animals had failed to gain, due to vit. A. deficiency, a single dose of 13.6 μg pure β-carotene† in aqueous Tween 40 was injected via tail vein. The food was continued *ad libitum* and the growth rate followed. In a *second similar study,* four groups of 6-7 rats were fed an A-free, low-tocopherol diet as above but containing 10% lard as a source of fat. From the 11th to 14th days, inclusive, the groups received 0, 0.01, 0.05 or 0.10 mg α-tocopherol by stomach tube. On the 15th day, they were injected intravenously with 12.3 μg carotene and the growth rates followed. In a *third study,* 2 groups of 4 rats were depleted of vit. A as in the first study. On the 13th, 14th and 15th days one group received 0 and the other 20 mg of α-tocopherol orally. On the 22nd day, when all animals were A-deficient (weights = 97-137 g), a single injection of 88 μg carotene/100 g body weight was given intravenously. Twenty-four hours later they were killed and analyses for carotene and vit. A(3) and tocopherol(6) performed.

Results. The data in Table I show that increasing the amount of α-tocopherol in the body of vit. A-deficient rats prior to injecting carotene did not affect the growth response to the carotene. All groups reached a weight plateau in 17-18 days and the growth curves were similar. In the second study, in which a low-vit. E diet was employed and much smaller supplements of α-tocopherol given the animals, again no effect of the tocopherol was observed on carotene utilization. The growth curves were similar and all groups reached a weight maximum in 12-14 days (average weight gains were 52.7, 55.3, 48.0 and 50.0 g for groups receiving 0, 0.01, 0.05 and 0.10 mg tocopherol, respectively. In both of these studies, negative control animals not receiving carotene failed to gain.

TABLE I. Maximum Weight Gains of Vit. A-Depleted Rats Dosed Orally with α-Tocopherol for 5 Days, then Injected Intravenously with 13.6 μg Carotene. (Max wt reached 17 or 18 days after receiving carotene.)

Daily α-tocopherol supplement, mg	No. of rats	Initial wt, g	Maximum wt, g	Wt gain, g
0	8	101.8 ± 4.6*	145.5 ± 6.6	43.7 ± 7.3
4	9	102.3 ± 2.4	150.4 ± 6.8	48.1 ± 5.3
10	10	102.7 ± 2.9	149.0 ± 8.2	46.3 ± 6.1
20	8	99.3 ± 3.4	148.8 ± 5.8	49.5 ± 3.6

* Mean with stand. error.

The formation of vit. A from carotene injected into rats with normal or elevated tissue levels of tocopherol is presented in Table II. The animals supplemented with α-tocopherol had considerably more of the anti-oxidant in their tissues than did the control animals. The amounts of vit. A formed, and the residual carotene, however, were essentially the same for the two groups.

Discussion. These studies indicate that when the tissue tocopherol level is increased above normal, and carotene is injected intravenously, no inhibition of carotene conversion occurs. Since carotene injected intravenously is transformed to vit. A predominantly in tissues other than the small intestine(3), it would appear that the inhibitory effect of tocopherol on orally administered carotene, under some conditions, is on the stability and absorption of the pro-vitamin in the intestine, and not on the actual enzymatic conversion to vit. A.

Summary. Increased amounts of tocopherol in tissues of rats did not affect the formation of vit. A from injected carotene, as

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† General Biochemicals, Inc.

TABLE II. Vit. A and Tocopherol in Tissues of Vit. A-Deficient Rats Injected with Carotene after Receiving Tocopherol Orally.

Tocopherol supplement	Liver + kidneys*		Tocopherol, μg/g†		
	Carotene, μg/g	Vit. A, μg/g	Heart	Lung	Testes
0	6.3 (5.3-8.2)‡	1.9 (1.7-2.2)	34.2 (30.4-40.2)	23.9 (20.8-29.6)	13.2 (11.5-15.5)
20 mg, 3×	6.7 (5.6-8.2)	2.1 (1.9-2.4)	40.2 (32.0-56.2)	32.5 (25.6-37.5)	18.6 (15.4-22.4)

* Avg of 3 rats. † Avg of 4 rats. ‡ Range of values.

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Received February 21, 1955. P.S.E.B.M., 1955, v88.

Effect of Purified Gonadotropins upon Release of Spermatozoa in the Frog, *Rana pipiens*.* (21627)

MARIO H. BURGOS† AND AARON J. LADMAN.‡ (Introduced by D. W. Fawcett.)

From Department of Anatomy, Harvard Medical School, Boston, Mass.

Houssay(1) reported that purified FSH, LH and human chorionic gonadotropin (HCG) stimulate the release of spermatozoa (spermiation) in the toad (*Bufo arenarum Hensel*). Since it was discovered that HCG could be assayed in this manner in various Salientia, the domestic frog (*Rana pipiens*) has been increasingly used as the assay animal in pregnancy diagnosis procedures(2-8). To our knowledge, the effects of purified pituitary gonadotropins on the spermatozoa release response in *Rana pipiens* have not been studied before. This report presents the results which we obtained with the 3 purified pituitary gonadotropins, FSH, LH and prolactin, and also with HCG.

Materials and methods. Frogs were procured from a local dealer during September and October, and placed in community tanks in which a constant flow of tap water was maintained. Doses of 0.5 mg to 15 mg of FSH, 0.005 mg to 1 mg of LH, 5 I.U. to 150 I.U. of prolactin (Armour)§ and 10 I.U. to 100 I.U. of HCG (Parke, Davis) in 0.5 ml of frog Ringer solution were prepared by serial dilutions and were injected into the dorsal lymph sac. Controls received 0.5 ml of Ringer solution alone. Subsequently, at the time of observation, a medicine dropper was inserted into the animal's cloaca. The fluid so withdrawn was examined unstained, under the microscope, for evidence of spermiation prior to and at intervals of ½, 1, 2, 3, 4, 24 and 48 hours after hormone administration. The magnitude of the spermatozoa release was evaluated and the response was recorded in the following terms: 0 (no re-

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* This study was supported by a grant from the Division of Research Grants and Fellowships of the National Institutes of Health, U. S. Public Health Service.

† Rockefeller Fellow 1953-1955.

‡ American Cancer Society Research Fellow 1952-1955.

§ We wish to thank Dr. Sanford Steelman of Armour Laboratories, for generous supplies of swine FSH No. K45308R, sheep LH No. 227-80, and beef prolactin No. R491153.

TABLE I. The Spermatozoa Release of the Frog *(Rana pipiens)* to 4 Gonadotropins.

Hormone	Dosage	Hr between hormone inj. and cloacal fluid examination	No. of animals inj.	No. of animals responding	% responding	Mean response value*
FSH	15 mg	½	2	0	0	.0
	15	1	2	2	100	1.5
	15	2–4	4	4	100	2.5
	5	2–2½	8	0	0	.0
	5	3–3½	8	7	87	1.3
	1	½–3	8	0	0	.0
	.5	3–3½	8	0	0	.0
LH	1	½–4	8	8	100	3.0
	.5	½–1½	12	12	100	3.0
	.5	2½–4	17	17	100	2.6
	.5	24	4	4	100	1.8
	.1	½–3	4	4	100	2.5
	.075	½–2	3	3	100	2.0
	.05	½	8	6	75	.8
	.05	1–1½	7	3	43	.4
	.05	2½–3	14	12	86	1.4
	.05	4	4	4	100	1.8
	.05	24	4	4	100	1.0
	.005	½–24	29	5	17	.2
Prolactin	150 I. U.	½–24	6	0	0	.0
	5	½–24	6	0	0	.0
HCG	100	2–3½	16	13	81	1.4
	100	24	3	3	100	1.0
	25	4	2	2	100	2.0
	10	2–3½	15	3	20	.3
	10	24	3	0	0	.0

* Mean response value computed by adding evaluated response scores of individual frogs (0, 1+, 2+, +3+) and dividing by total No. of animals in group.

sponse), 1+ (weak), 2+ (moderate) and 3+ (strong). *Testes* from control and stimulated frogs were fixed in Bouin's fluid, dehydrated, embedded in paraffin, and sectioned at 4μ. The sections were stained in hematoxylin and eosin.

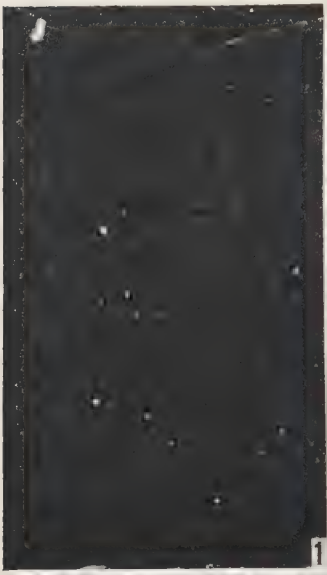
Results. The responses of the frogs to administration of the 4 gonadotropins, as measured by the release of spermatozoa, are presented in Table I. The 3 photographs shown in Fig. 1 to 3 illustrate typical responses, as observed microscopically in cloacal washings.

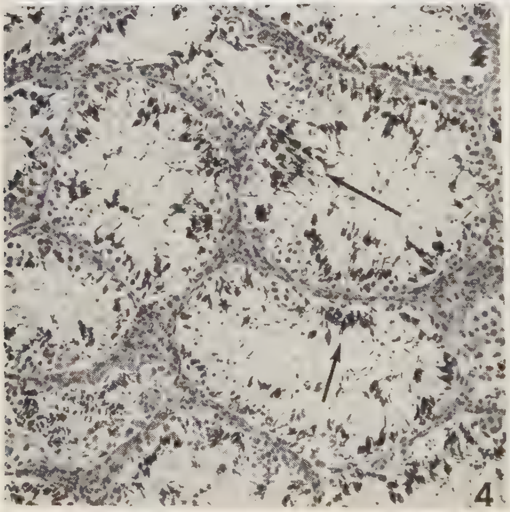
The minimal doses of hormone which were necessary to elicit the release of spermatozoa in greater than 75% of the frogs in each group within 4 hours after administration were: 5 mg of FSH, 0.05 mg of LH and 25 I.U. of HCG. Prolactin failed to elicit a response. Only occasional responses were observed after administering 0.005 mg of LH and 10 I.U. of HCG. The extent of spermiation as determined by the mean response value in Table I appears to be maximal at 4 hours, diminished after 24 hours, and negative by 48 hours after injection. When doses of 0.1 mg of LH, or more, were given, maximal responses were observed within 20 minutes. We reused these animals after 48 hours and observed no cumulative effects.

The histological appearance of the frogs' testes prior to and ½ hour after administration of 0.1 mg of LH is shown in Fig. 4 and 5. In control animals (Fig. 4), heads of the spermatozoa lie clustered in groups, and buried in the apical cytoplasm of the Sertoli cells at the periphery of the seminiferous tubules (arrows). After strong stimulation (Fig. 5), the spermatozoa and a few dislodged Sertoli cells are forced into the center of the tubules, whereas the spermatogonia and spermatocytes remain as rounded clusters of cells which are adherent to the basement membrane of the tubules.

Discussion. Spermiation in frogs can be induced by FSH, LH, and HCG, but not by prolactin. Nevertheless, it should be noted

FIG. 1-3 are dark-field photomicrographs of unstained cloacal washings of frogs to illustrate spermatozoa release response. All ×100.





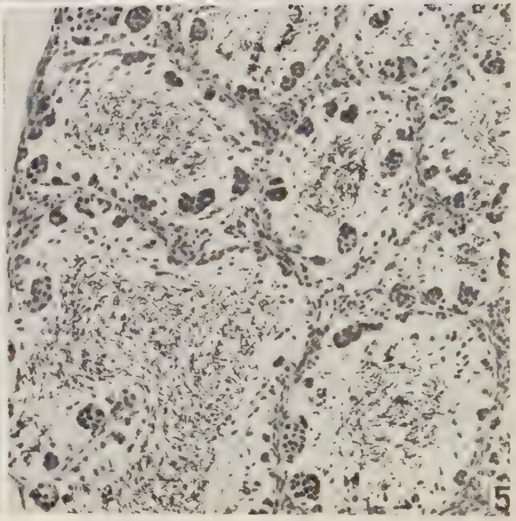


FIG. 1. Control animal. No spermatozoa are present.

FIG. 2. A 1+ response. Small number of spermatozoa, which resemble grains of rice, are seen.

FIG. 3. A 3+ response. Large numbers of spermatozoa clustered about Sertoli cells which have been expelled with spermatozoa by intensity of the stimulation.

FIG. 4. A photomicrograph of a histological section of the testis from a control frog. The spermatozoa heads (arrows) are seen at periphery of seminiferous tubules implanted in cytoplasm of the Sertoli cells. H. & E. ×110.

FIG. 5. Photomicrograph of histological section of testis from a frog which displayed a 3+ response, 30 min. after administration of 0.1 mg of LH. Spermatozoa and some Sertoli cells are visible in centers of seminiferous tubules, whereas, spermatogonia and spermatocytes are collected in rounded groups which are adherent to the basement membrane enclosing the tubules. H. & E. ×110.

that the preparation of FSH contains 2 to 3% of LH as determined by the manufacturer's assay (weight of ventral prostate in hypophysectomized immature rats), and that the minimal dosage of 5 mg of FSH which evoked a positive response, contained by calculation, 0.1 mg to 0.15 mg of LH. This amount of LH, as shown in Table I, is sufficient to elicit spermiation. Similarly, the doses of 1 mg and 0.5 mg of FSH which did not evoke spermiation, contained only 0.02 mg to 0.03 mg, and 0.01 to 0.015 mg of LH, respectively, amounts which are less than the minimal 0.05 mg dose of LH required to induce a uniformly positive response. Thus, we attribute the spermiation response which occurred after administration of moderately large doses of FSH to the LH with which the preparation was contaminated. As a result of these considerations, it is concluded that the observed release of spermatozoa in the frogs was induced solely by LH or a substance with an LH-like activity, such as HCG. Since this method for the determination of LH activity as measured by spermatozoa release in frogs is rapid, sensitive and relatively inexpensive, it might be utilized as an accurate procedure to assay the purity of FSH preparations.

Occasional positive responses in *Rana pipiens* after the administration of menopausal urine were attributed by Haskins(9) to FSH, an interpretation which we do not share. From our experience with FSH, we believe the presence of LH in menopausal urine to be a more likely explanation. The finding of Greep and Jones(10) that, in addition to large quantities of FSH, menopausal urine contains appreciable amounts of LH, lends strong support to the latter supposition.

The possibility that spermiation can be induced in *Rana pipiens* by the release of epinephrine, as a result of handling the animals, has been suggested by Robbins and Parker (11). Since the controls as well as the experimental animals which received nonspermiating doses of LH and HCG did not respond, the possibility that epinephrine may induce spermiation appears to be ruled out under the conditions of our experiments.

Because of the delicate and rapid response of the germinal elements of the testis to some of these preparations, further study of the experimental material by means of histological and cytochemical technics suggested itself. This work is now in progress and the results will be published subsequently.

Summary. Release of spermatozoa by the frog, *Rana pipiens*, after administration of "purified" FSH, LH, prolactin and HCG has been studied. The minimal doses of these hormones which produced a response in over 75% of the animals in each group were: 5 mg of FSH, 0.05 mg of LH and 25 I.U. of HCG. Prolactin did not elicit a response. Our data indicate that the release of spermatozoa in the frog can be induced by LH or a substance with an LH-like activity (HCG). The positive response to FSH is attributed by us to the contamination of the preparation with LH (2 to 3% by manufacturer's assay). It is suggested that this rapid and sensitive response to LH might be utilized to assay the purity of FSH preparations.

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Received February 23, 1955. P.S.E.B.M., 1955, v88.

Failure of Cobalt to Influence the Life Span of the Erythrocyte. (21628)

D. C. VAN DYKE,* C. W. ASLING,† N. I. BERLIN,‡ AND R. G. HARRISON.

From Department of Anatomy, University of Liverpool, National Institute of Medical Research, London, and The Donner Laboratory, University of California, Berkeley.

Chronic administration of cobalt is one of the few ways in which an increase in the total number of erythrocytes in the circulation (polycythemia) can be produced. Since polycythemia must result either from an increase in the rate of formation of erythrocytes or from an increase in the average life span of the erythrocytes, or a combination of these two, it is important to determine which mechanism is involved following the administration of cobalt(1). It is generally believed that cobalt acts as a direct stimulant to the erythropoietic portion of the bone marrow since its administration is followed by a reticulocytosis(2), hyperplasia of the bone marrow(3,4) and an increase in the erythroid cells in the marrow(5-8). Studies of iron metabolism in the rat made polycythemic by cobalt showed that there was an increase in the amount of iron utilized for red cell formation which was directly proportional to the increase in total red cell volume(9). This could be interpreted as meaning that the life span of the red cell was normal. Nevertheless, one must consider the possibility that an increase in red cell life might be contributing to the final degree of polycythemia. Therefore, a direct measurement of the life span of the red blood cell in the rat made polycythemic by cobalt should be made. In this study the life span of the erythrocyte in such rats was determined by the method of Shemin and Rittenberg(10) using C^{14} labeled glycine.

Materials and methods. Female albino rats (Imperial Chemical Industries colony), 90 days of age, averaging 150 g, were distributed between 2 groups. One group (8 animals) received daily 1.5 ml intraperitoneal injections of a mixture of cobaltous sulfate and ferric ammonium citrate containing 0.187 mg cobalt and 0.3 mg iron. The iron was included to provide for any increase in hemoglobin synthesis produced by cobalt. The second group (10 animals) received no treatment and served as controls. Previous experience has shown that this dose and route of administration, if given for an adequate period of time produces a true polycythemia(11). When the cobalt-injected group had developed a significant polycythemia, as judged by the hematocrit (94 days after the onset of cobalt therapy), the animals of both the cobalt-injected and uninjected groups were given intraperitoneally 0.1 mg glycine containing 5 μc of glycine-2-C^{14}. After administration of the glycine, 0.25 ml samples of blood were drawn from the tail veins at frequent intervals for the next 83 days. During this period the cobalt and iron injections were continued and the polycythemia was maintained as demonstrated by hematocrit readings at the end of the experiment. This period of sampling (83 days) exceeded the normal life span of the rat's red cells (60 days)(12). In order to minimize blood loss through sampling and yet allow frequent sampling, only half of the members of each group were bled at each interval. The blood was drawn into a heparinized syringe and the cells were washed twice with physiologic saline solution. The hemoglobin was extracted by a modification of the method of Drablin (13), oxidized by the method of van Slyke (14), and the liberated CO_2 collected as $BaCO_3$. The C^{14} activity of the CO_2 subsequently liberated from a known amount of $BaCO_3$ by treatment with concentrated sulfuric acid was measured in a 100 cc ionization chamber(15).

Results. The Table gives the weight of the animals and the hematocrits at the time of administration of glycine-2-C^{14} and also at the end of the experiment. It will be seen

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[*] Present address: Institute of Experimental Biology, University of California, Berkeley.

[†] Fulbright Fellow, Department of Anatomy, University of Liverpool.

[‡] U. S. Public Health Service Special Research Fellow, National Institute for Medical Research, London.

TABLE I. Body Weight and Hematocrit Measurements at Time of Injection of C^{14} Labeled Glycine and at End of Experiment.

Group	No. of rats	At onset Body wt, g	 Hematocrit, %	Terminal Body wt, g	 Hematocrit, %
Cobalt-inj.	8	193 ± 3.7	61.1 ± 1.11	223 ± 3.5	58.8 ± 1.22
Control	10	205 ± 2.6	47.0 ± .78	231 ± 3.2	43.7 ± .91

that the weight gain was impaired only slightly by this dose of cobalt and also that the hematocrits remained constant during the experiment in spite of the loss of blood involved in sampling.

The graph shows the average values for the radioactivity of the carbon derived from hemoglobin, expressed as disintegrations per minute per mg $BaCO_3$ at progressive intervals after the injection of labeled glycine in both the polycythemic and normal rats. It should be noted that the specific activity for the polycythemic animals is approximately 60% greater than that of the normal animals (8 *vs* 5 disintegrations per minute) in the initial samples. This higher level results from a greater incorporation of glycine into the red cells of the polycythemic animals. The most likely interpretation for this greater incorporation is that it results from an increased rate of formation of red cells at the time the glycine was injected. The points of inflection obtained by graphical differentiation of the 2 curves indicate the average life span of the red cell(10). The average red cell life of 53 days found in the normal animals is in agreement with previous estimates(12). The life span of the red cells of the animals made polycythemic by cobalt was not different from that of the normal controls.

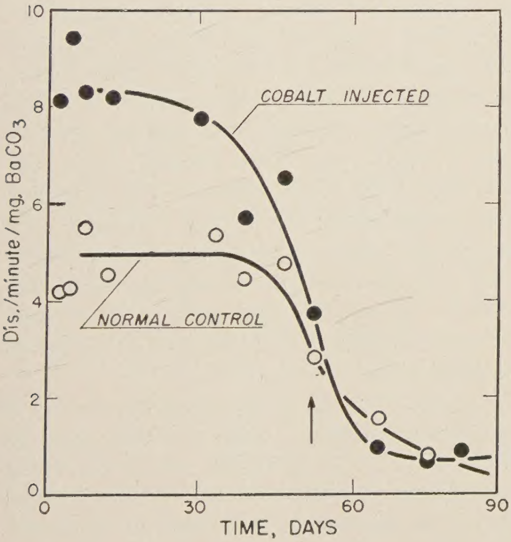

FIG. 1. Values for avg radioactivity of carbon derived from hemoglobin are expressed in disintegrations/min./mg $BaCO_3$. Point of inflection, indicating avg life of erythrocyte is shown by an arrow at 53 days.

Conclusions. This study shows that the life span of the red cell is not changed in rats made polycythemic with cobalt. The mechanism for the production of polycythemia following chronic administration of cobalt must be attributed to an increase in the rate of production and release of red cells into the circulation.

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Received March 2, 1955. P.S.E.B.M., 1955, v88.

Recovery of Western Equine Encephalomyelitis Virus from Naturally Infected English Sparrows of New Jersey, 1953.* (21629)

PRESTON HOLDEN.[†] (Introduced by W. McD. Hammon.)

From the Department of Epidemiology and Microbiology, Graduate School of Public Health, University of Pittsburgh, Pittsburgh, Pa.

The virus of western equine encephalomyelitis (WEE) has been recovered numerous times from both vertebrate and invertebrate hosts in the middle and far western states of the United States. Relatively few isolations from naturally infected hosts have been reported from states east of the Mississippi River, and none east of the Appalachian Mountains. This communication describes one definite and one probable recovery of WEE virus from immature English sparrows (*Passer domesticus domesticus*) found in New Jersey in September, 1953. Further evidence that the virus was active among avian hosts of the area was found in a WEE antibody survey of domestic fowl.

Methods. English sparrows were collected in wire bait traps placed one mile west of Egg Harbor City, New Jersey, as a part of an investigation of eastern equine encephalomyelitis (EEE) in ring-necked pheasants (*Phasianus colchicus torquatus*) (1). Blood specimens were taken from the external jugular vein, placed on wet ice for transportation to the field laboratory, and subsequently stored on dry ice either as whole blood or serum. Whole blood specimens were prepared for attempted virus isolation by grinding 0.3 to 0.5 ml amounts of mixed clot and serum in a cold mortar with alundum and 3.0 ml quantities of diluent consisting of 33⅓% normal inactivated rabbit serum in buffered saline and containing penicillin and streptomycin in final concentrations of 667 units/ml and 667 μg/ml, respectively. Following centrifugation at 2,000 rpm for 20 minutes at 5°C, 0.03 ml amounts of the supernatants were inoculated into each of six 3- to 4-week-old mice (CFW) by the intracerebral route and 0.09 ml by the intraperitoneal route. Specimens consisting of serum alone were diluted with equal amounts of serum antibiotic mixture and inoculated by the intracerebral route only. The brains of sick mice were removed aseptically and used for the preparation of stocks for further passage. In mouse neutralization tests used for identifying viruses or for detecting humoral antibodies, constant amounts of serum or plasma were mixed with serial 10-fold dilutions of virus. After incubation at 37°C for 2 hours in a water bath, the serum-virus mixtures were inoculated by the intracerebral route. Neutralization indices were computed by standard methods (2). In November, 1953, an antibody survey was made of domestic fowl reared on the Egg Harbor City pheasant farm and on another farm located near New Gretna, N. J., approximately 10 miles distant. For this purpose, heparinized blood was obtained from the jugular vein. Plasma was separated by slow speed centrifugation and stored at approximately −20°C until used in neutralization tests against a standard laboratory strain of WEE virus.

Results. In the virus isolation studies, 2 agents were obtained that were identified as WEE virus. One of these, strain NJ 1044, was recovered from a whole blood specimen drawn on September 11 from an immature English sparrow. The second, strain NJ 1097, was isolated from pooled sera obtained on September 7 from two immature birds of the same species. The results of neutralization

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* This and related studies provided the basis for a thesis submitted by the author to the Graduate School of Public Health of the University of Pittsburgh in partial fulfillment of the requirements for the degree of Doctor of Public Health. The investigation was sponsored jointly by the Communicable Disease Center, Public Health Service, U. S. Department of Health, Education and Welfare; the New Jersey State Department of Health; the Graduate School of Public Health, University of Pittsburgh; and the Commission on Virus and Rickettsial Diseases, Armed Forces Epidemiological Board.

[†] Communicable Disease Center, Public Health Service, U. S. Department of Health, Education and Welfare, Atlanta, Ga.

TABLE I. Results of Neutralization Tests Used to Identify WEE Viral Isolates Obtained from English Sparrows Trapped in New Jersey, 1953.

Laboratory strain No.		E. sparrow specimen	LD$_{50}$ Normal serum	LD$_{50}$ WEE immune serum	LD$_{50}$ EEE immune serum	Neutralization index WEE immune serum	Neutralization index EEE immune serum
NJ 1044	P-3*	Whole blood	7.3	4.3	6.3	1000	10
NJ 1044 (repeat)	P-2*	" " †	6.4	3.0	5.5	2520	8
NJ 1097	P-4*	Pool of 2 sera	6.0	3.0	6.0	1000	1

* Denotes No. of mouse passages.

† Stored duplicate of whole blood specimen from which the NJ 1044 P-3 strain was originally recovered.

tests used to identify each isolate are given in Table I. In these tests, neutralization indices of 1,000 or greater were obtained when WEE immune serum was used against the unknown strains; EEE immune serum did not neutralize to a significant degree. The identity of the NJ 1044 strain was further confirmed by the results of vaccination-challenge tests. Two guinea pigs, immunized against a standard strain of WEE virus, remained normal following intracerebral challenge with 0.2 ml of a 10^{-2} dilution of a third passage pool (greater than 100,000 mouse LD$_{50}$ doses). A control guinea pig of the same age developed typical signs of encephalitis and was sacrificed *in extremis* on the third day. The agent was later recovered from its brain by mouse inoculation.

Data obtained in studies of the NJ 1044 isolate strongly suggest that it represents a definite recovery of WEE virus from a naturally infected sparrow. Of the 6 mice used for original isolation of this strain, one was sacrificed on the third day and one on the fifth day following inoculation. Three mice died, one on each day from the fourth to the sixth. The remaining mouse was not visibly affected. On serial passage using 10^{-1} and 10^{-2} mouse brain suspensions, the agent adapted readily to mice, the second passage causing advanced signs of disease in all mice on the third day following inoculation. The presence of virus in the original whole blood specimen was later confirmed by mouse inoculation of a frozen duplicate portion. This material was prepared for inoculation in 10-fold serial dilutions. Of 10 mice inoculated with the lowest dilution (approximately 10^{-1}), 5 deaths occurred on the third day after inoculation, 3 on the fourth, and one on the fifth, a total mortality of 9 out of 10. Twenty mice were inoculated with the 10^{-2} dilution. Of these, one died on the third day, one on the fifth, 4 on the sixth, and one on the tenth, a total mortality of 7 out of 20. The agent recovered from brains of these mice (NJ 1044 (repeat) P-2) was also identified as WEE virus by neutralization tests (Table I).

The recovery of WEE virus from sparrow serum (NJ 1097) was not completely confirmed. Of the 6 mice originally inoculated with this specimen, one was observed to be sick on the sixth day and its brain was harvested for further passage. The remaining 5 mice were not observed to show signs of disease at any time. The agent isolated was adapted to mice with considerably more difficulty than was the NJ 1044 strain, the incubation period being longer and more irregular in early passages. The second and third passages caused deaths with greatest frequency on the fourth and fifth days following inoculation. Four passages were required before deaths occurred uniformly on the third day. The LD$_{50}$ titer of the fourth passage stock in the presence of normal serum was $10^{-6.0}$ as compared with $10^{-6.4}$ for the NJ 1044 (repeat) strain in second passage and $10^{-7.3}$ for the NJ 1044 strain in third passage (Table I). On the 28th day following the original isolation, an unsuccessful attempt was made to recover the agent from a frozen duplicate specimen. This was not unexpected in view of the longer period of storage and because only one of six mice was visibly affected in the earlier test.

TABLE II. Results of WEE Virus-Neutralization Tests on Plasma Obtained from Domestic Birds, New Jersey, 1953.

Domestic birds tested*	Results: Negative N.I. <20	Equivocal N.I. 21-50	Positive† N.I. >50	Total tested
New Gretna				
Immature pigeons	4	0	0	4
" chickens	17	0	0	17
" pheasants	16	0	3	19
2-year-old pheasants	4	0	9	13
" " " mallard ducks	2	0	0	2
Egg Harbor City				
Immature pheasants	31	2	0	33
Total	74	2	12	88

* Immature birds hatched in the spring of 1953.
† Positive indices in range between 74 and 1000.

In WEE virus-neutralization tests on 88 plasma specimens obtained from domestic birds of known ages, 12 were considered positive and 2 were considered equivocal (Table II). Of the positive tests, a neutralization index of 74 was obtained in one, all others were in the range 100 to 1000. All positive and equivocal plasma specimens were obtained from pheasants.

Discussion. An accepted belief of most investigators of the viral encephalitides is that, in the United States, WEE is endemic only in the middle and far western states. The results of this study give indication that WEE virus was active among avian hosts in New Jersey during 1952 and 1953. Since immature English sparrows rarely, if ever, migrate long distances, it is highly improbable that the birds from which virus was isolated in 1953 had become infected outside of New Jersey. In the WEE antibody survey, the high proportion (9 of 13) of positive tests among two-year-old pheasants and the low proportion (3 of 19) among immature birds in the same flock suggest that infection had occurred both in 1952 and 1953.

There is considerable evidence that the 2 virus isolations from sparrows did not result from laboratory contamination. A careful search of the records revealed that no strain of WEE virus was in use in the laboratory at the time of or during the month preceding the first positive isolation attempt. In addition, the laboratory strains uniformly cause deaths in mice between 36 and 48 hours after intracerebral inoculation as contrasted with the three to six days required by first mouse passage stocks of the 2 isolated agents. Although the initially isolated WEE virus was under test at the time of the second isolation, the differences in behavior of the two strains in mice suggest that each is a separate and distinct entity. Further support for the isolation of the NJ 1044 strain and evidence that it was not a laboratory contaminant was obtained by reisolation of virus from the remaining portion of the original blood specimen.

Summary. 1. One definite and one probable isolation of western equine encephalomyelitis (WEE) virus are reported from whole blood and serum, respectively, of apparently normal immature English sparrows trapped in New Jersey in September, 1953. 2. Neutralizing substance against WEE virus was found in 12 of 88 plasma specimens obtained from domestic fowl of the same general area.

Grateful acknowledgement is made to members of the faculty and technical staff of the Graduate School of Public Health, University of Pittsburgh, for advice and technical assistance. Special acknowledgement is made to Dr. William McD. Hammon for guidance and assistance throughout the study.

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Received February 25, 1955. P.S.E.B.M., 1955, v88.